DOCTOR OF PHILOSOPHY

Hormone-induced expression of the epithelial sodium channel in human airway cells

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Hormone-Induced Expression of the Epithelial Sodium Channel in Human Airway Cells

Noor Ismail

2013

University of Dundee

HORMONE-INDUCED EXPRESSION OF THE EPITHELIAL SODIUM CHANNEL IN HUMAN AIRWAY CELLS

Noor A S Ismail

A thesis submitted as a part of fulfilment in Doctor of Philosophy

Centre of Cardiovascular and Lung Biology
University of Dundee

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List of Abbreviations

4EBP1	eukaryotic initiation factor 4E binding protein
AGC kinases	Protein Kinase A / Protein Kinase G / Protein Kinase C family
ANOVA	Analysis of Variance
AQP5	Aquaporin 5
ASIC1	acid-sensing ion channel 1
ATI	alveolar type I
ATII	alveolar type II
BCS	Bovine Calf Serum
cAMP	cyclic Adenosine Monophosphate
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
Cl^-	chloride ion
ClC	chloride channel family
CNG	Cyclic Nucleotide-Gated channel
CO_2	carbon dioxide
COOH	carboxylic acid
CREB	cAMP Response Element Binding Protein
C-terminal	carboxylic terminus
db-cAMP	dibutyryl cyclic adenosine monophosphate
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl Sulphoxide
EC_{50}	Concentration for 50% activation
ENaC	Epithelial Na^+ Channel
FBS	Foetal Bovine Serum
FDLE	foetal distal lung epithelium
FRT	Fischer rat thyroid
GC	glucocorticoid
G_{Na+}	Na^+ conductance
GRE	glucocorticoid response elements
H_2O	water/fluid
H_2O_2	hydrogen peroxide
HRP	Horse Radish Peroxidase
Hc	hydrocortisone

IP	Immunoprecipitation
Li^+	Lithium ion
MDCK	Madin-Darby canine kidney cell
mpk-CCDc14	mouse collecting duct principle cell line
mTOR	Mammalian Target of Rapamycin
mRNA	messenger Ribosomal Nucleic Acid
mSin1	mammalian Stress activated protein kinase Interacting protein 1
Na^+	Sodium ion
Na^+-K^+-ATPase	sodium potassium Adenosine Triphosphate channel
NDRG1	N-myc Downstream Regulated Gene 1
Nedd4-2	Neural Precursor Cell-Expressed Developmentally Down-Regulated Protein 4
NH_2	amino acid
N-terminal	NH_2 terminus
NSCC	nonselective cation channel
NKCC	sodium, potassium, chloride channel
O_2	oxygen
P2Y2	P2Y purinoceptor 2
P70-S6K	70 kDa Ribosomal S6 Kinase
PBS	Phosphate Buffered Saline
PCO	partial pressure of carbon dioxide
PDK1	Phosphoinositide-dependent Protein Kinase 1
PHA	pseudohypoaldesteronism
PI3-Kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 3,5 – biphosphate
PIP_3	Phosphatidylinositol 3,4,5 – biphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PO_2	partial pressure of oxygen
PRAS40	Proline-rich Substrate of Akt - 40 kDa
PY motif	Proline-rich motif
RDS	Respiratory Distress Syndrome
S6K1	P70-S6 kinase 1
SDS	Sodium Dodecyl Resistance
SEM	Standard Error of Mean
SGK1	Serum-and Glucocorticoid-Regulated Kinase 1

T_3	Triiodothyronine
TBS	Tris-buffered Saline
TBS-T	Tris-buffered Saline with Tween
TEMED	Tetramethylethylenediamine
TOR	target of rapamycin
TORC1	target of rapamycin complex 1
TORC2	target of rapamycin complex 2
WW	proline-motif protein

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Declaration

I hereby declare that this thesis entitled *"Hormone-induced expression of the epithelial sodium channel in human airway cells"* has been prepared by me under the direct guidance of Dr. Stuart M. Wilson and Dr. Stephen C. Land as part of the work required for the award of PhD in Lung Biology at the University of Dundee, Dundee, Scotland.

I have not submitted this thesis previously for the award of any degree or diploma at any other institution. The work recorded throughout this study has been carried out by myself, unless otherwise stated, and all other sources of information have been acknowledged where appropriate.

22/08/2012

Supervisor's Statement

We certify that Noor A S Ismail has fulfilled the conditions of ordinance 39; within the regulations she is qualified to submit this thesis in application for the higher degree of Doctor of Philosophy.

Dr. Stuart M Wilson

Dr. Stephen C Land

Abstract

Respiratory distress syndrome and pulmonary oedema often result in poor health and in the worst case scenario, death. Several studies have proposed that the eventual resolution of these dangerous conditions is due to active sodium reabsorption through the epithelial sodium channel (ENaC), which is crucial for lung fluid clearance. Although clinical prognosis can be improved by using glucocorticoid hormones to augment the ENaC-dependent removal of liquid from the lungs, we still require a better understanding of the underlying mechanism in order to improve treatments in the future. This thesis, therefore explores the role of serum / glucocorticoid-inducible protein kinase 1 (SGK1) and protein kinase A (PKA) in the responses of hormone-stimulated H441 human airway cells. Dexamethasone, a synthetic glucocorticoid hormone, is thought to evoke expression of the gene encoding SGK1 and, to become catalytically active, this gene product must then be phosphorylated via TORC2 and PDK1, protein kinases activated via the P13-kinase pathway. Once activated, SGK1 appears to exert control over the surface abundance of ENaC subunits by phosphorylation, and thus inactivating, a ubiquitin ligase (Nedd4-2), that normally mediate the withdrawal of ENaC subunits from the plasma membrane. Protein kinase A (PKA) may contribute to this control mechanism by also phosphorylating Nedd4-2. In order to clarify the way in which these pathways contribute to glucocorticoid-induced lung liquid clearance, the present thesis has explored the effects of dexamethasone and / or PKA activation upon the overall / surface expression of ENaC subunits, the activities of SGK1 and PKA and the phosphorylation status of physiologically-important residues within Nedd4-2 itself.

1

Introduction

1.1 The respiratory system

The human body consists of cells that can replicate, defend and maintain the optimum condition. All cells require energy to conduct this work, and this is primarily derived from oxidative metabolism and this, in turn, implies that oxygen (O_2) must be taken into the body. Moreover, the carbon dioxide (CO_2) produced by respiring tissues must also be eliminated, and multicellular organisms therefore need specialised structures to permit gas exchange. Organisms that live in water achieve this either via direct diffusion across the body surface or via specialist structures known as gills (e.g. fish, amphibian larvae, crustaceans). On land, this function is fulfilled by the lungs. The exchange of gases takes place across the alveolar epithelium which is very thin in order to allow gas exchange (Boucher, 1999). Since the body can require considerable amounts of oxygen, a large surface area must be available for gas exchange and, in an adult human, the overall area of the alveolar epithelium has been estimated to 70-140 m^2, which is 35 times the surface area of the body (Martini, 2004a). Therefore, the

development of the structures of the lung was vital for the evolution of large complex organisms. However, the respiratory system does not simply act as a gas exchange region, it also accounts for many problems which the lung has evolved to overcome.

1.1.1 Gas exchange

The primary function of the respiratory system is to serve as a site for gas exchange. However, this system must be initiated by breathing. Air is inspired from the atmosphere through the nose and passes through the nasopharynx which performs the vital function of warming and humidifying the air. This process is crucial to preserve the optimal body temperature (37^{o}C), otherwise enzymatic function could not be maintained. The inspired air has a much lower humidity than air in the respiratory system, thus without being humidified, water vapour would move into the air from the lining of the respiratory system, thereby reducing airway surface hydration. When the inspired air reaches the alveolar lung for gas exchange, the pressure of O_2 (~100 mmHg) is lower than the atmospheric O_2 (~159 mmHg). This is due to the partial pressure of O_2 (PO_2) in the alveoli as a result of the combination of the O_2 uptake and supply. In parallel, the pulmonary artery carries blood which has a low concentration of O_2 (O_2 is ~40 mmHg) to the lungs, therefore the difference in pressure will result in diffusion. However, O_2 must first dissolve into the fluid lining of the alveoli before it can move via diffusion into the blood. This acts as a barrier which coordinates well with Fick's law of diffusion; the net rate of diffusion of a substance is inversely proportional to the thickness of what it must cross. This indicates the importance of the liquid lining of the alveoli. This can be marked by the control of its depth; if the depth were to increase, then the time taken for O_2 to move into the blood would greatly increase and could lead to insufficient gas exchange. The reverse is true for CO_2 where the partial pressure of CO_2 (PCO_2) is higher in the blood than in the alveoli and CO_2 is expelled. Therefore, tight regulation of alveolar hydration is vital to the healthy function of the respiratory system. If this hydration is not controlled properly it can lead to excess fluid in the

lung, which has been shown to cause inefficient gas exchange (Berger *et al.*, 1996) and is a contributory factor in respiratory distress syndrome (RDS), a leading cause of death in premature and newborn infants (O'Brodovich, 1996).

1.1.2 Development of the respiratory system

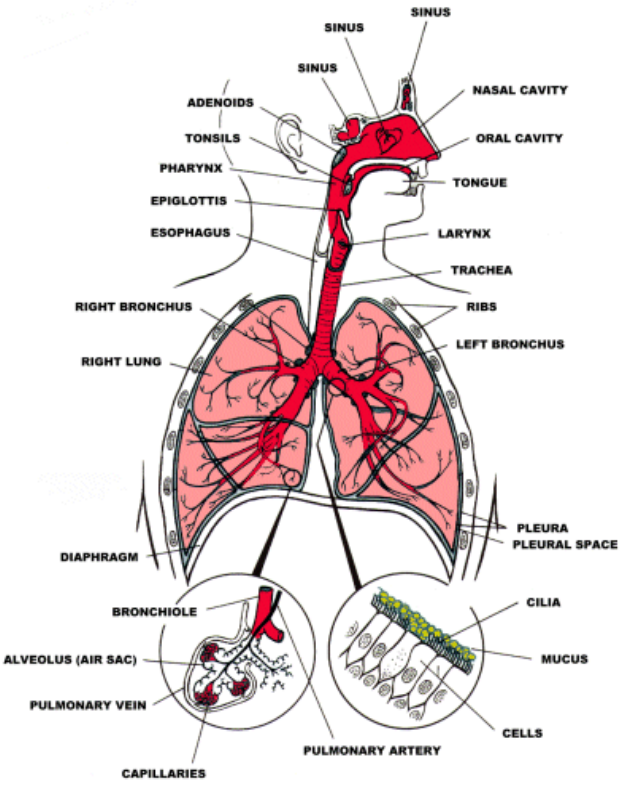

Figure 1-1: Schematic diagram of the human respiratory system (www.lung.ca/respiratory_system)

The respiratory system begins to develop after the fourth week of gestation (Boyden, 1977). When considering the development of this system it is important to review the development of the foregut, and the development of the endoderm overall. It is also important to consider the development of the respiratory system in terms of its many constituent components. The respiratory system commences at the nasal cavity and consists of a conducting portion and a respiratory portion. The conducting portion includes the nasal cavity, pharynx, larynx, trachea,

bronchi, and bronchioles (*Figure 1-1*). The respiratory portion consists of the alveolar ducts, alveolar sacs and the alveoli, and gaseous exchange occurs in the alveoli.

The development of the respiratory system involves the endoderm and the mesoderm that surrounds it. First, the embryo undergoes lateral body folding and during this process the endoderm forms into a gut tube. This gastrointestinal tube begins cephalically at the oral plate and continues until it reaches the anal plate. At the time the body folding is completed, the gut tube can be subdivided into three divisions: a foregut, a midgut and a hindgut (O'Rahilly *et al.*, 2004). The larynx is first seen as an outgrowth from the foregut. This outgrowth of tissue is called the respiratory diverticulum or the lung bud. The formation of the lung bud occurs when two lateral folds of mesoderm and endoderm meet at the midline and separate the larynx and trachea from the oesophagus. The lung bud is a ventral diverticulum of the endoderm that arises from the floor of the foregut caudal to the pharynx. The diverticulum forms a groove in the floor of the pharynx called the laryngotracheal groove (O'Rahilly *et al.*, 2004).

The trachea then develops caudal to the larynx. The epithelium develops from the endoderm and the tracheal cartilage and muscles develop from the mesoderm. Early in development the trachea bifurcates into the left and right bronchi (Haefeli-Bleuer,1988). As the bronchi develop they continue to branch. The right bronchus gives off three lung buds and the left bronchus gives off two lung buds (*Figure 1-2*). These lung buds become the lobar bronchi and indicate that the right lung will have three lobes and the left lung will have two lobes (Bucher *et al.*, 1961). Each of the bronchi at this stage will divide into smaller bronchi. The branching of the bronchi continues until the bronchioles begin to form. In all there are 17 divisions of the bronchi until the sixth foetal month is reached (Bucher *et al.*, 1961). However, by early childhood there will be a total of 24 generations of branching as seen in Figure 1-3 (Harding & Hooper, 1996).

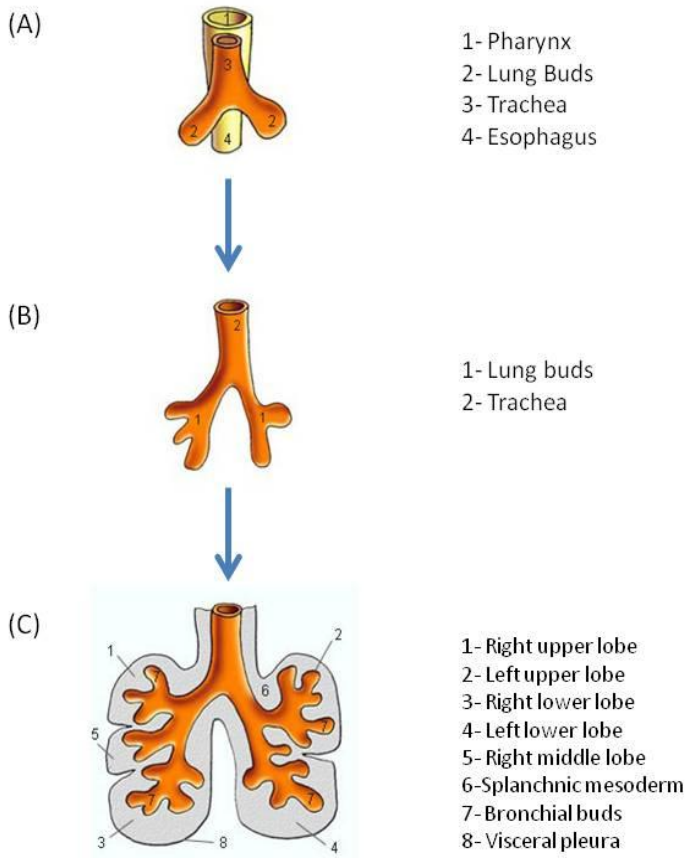

Figure 1-2: Schematic diagram of human lung formation (http://www.embryo.chronolab.com).

(A) The lung buds[2] start to branch out from the trachea to become a bronchus. *(B)* The right bronchus will further branch out to form three lung buds[1] whilst the left bronchus gives off two. *(C)* The lung buds will become bronchial buds[7] within the characteristic inner layer of splanchnic mesoderm[6]. There are three lobes in the right lung and two lobes in the left lung in the delicate serous membrane of the visceral pleura[8].

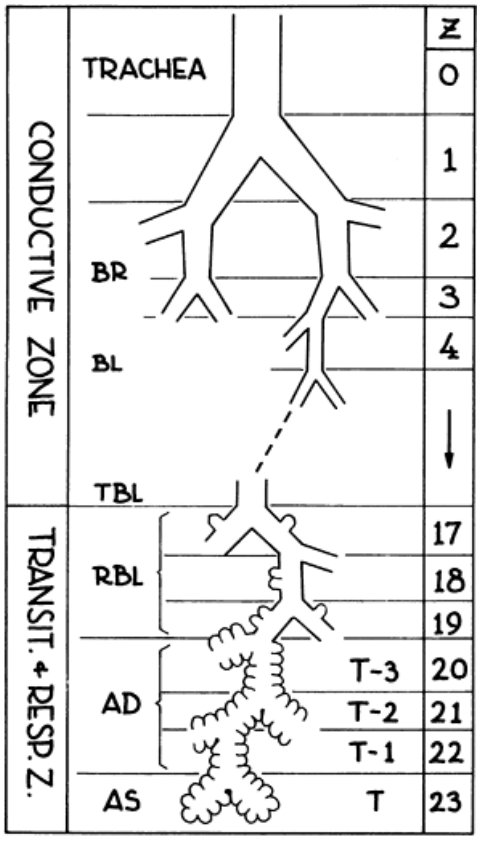

Figure 1-3: **Schematic representation of the sequence of airway branches** as a function of generation z. Bronchi (BR), bronchioles (BL), terminal (TBL), and respiratory (RBL) bronchioles are followed by alveolar ducts (AD) and sacs (AS) in the terminal generation. T=23 (Weibel, 1963).

As the lungs develop and divide into smaller divisions there are changes to the vascular supply of the lungs. The lungs can be described as undergoing 4 phases of development (Figure 1-3). During the first phase of development, the pseudoglandular period, the bronchi divide into smaller and smaller branches, the bronchioles (Wiebel *et al.*, 1963). This period occurs from the 2nd month through to the end of the 4th month. During the next 2 and 1/2 months the respiratory bronchioles are formed. They will give rise to alveolar ducts. This is called the canalicular period. During this time period the epithelium remains as a cuboidal epithelium and the capillaries, while proliferating, begin to approach the respiratory epithelium.

The next phase of development occurs from the 7th month until birth. During this period, the terminal saccular, the number of capillaries increases and they approach the respiratory epithelium. At the same time the terminal sacs form (Zeltner & Burri, 1987). This results in the formation of a squamous epithelium made up of type I alveolar epithelial cells, which will permit gaseous exchange. Hence, from the 7th month on the foetus is capable of survival. In the 7th month the type II alveolar epithelial cells also start to develop. These type II cells produce surfactant**,** the fluid that reduces the surface tension at the alveolar cell surface (Mason *et al.*, 1991). Finally, from the 8th month onwards, the mature alveoli continue to be formed with an increase in the amount of surface area where capillaries and alveolar cells are in contact. The functional alveolar epithelial cells appear in the prospective alveoli to differentiate between the type I and type II-like phenotype by flattening and increasing the epithelial surface to create the gaseous barrier (Schneeberger, 1991). This is eventually marked by the development of fully mature alveoli. This period of lung development is the alveolar period and actually can last through to the age of ten. The growth of the lungs after birth is mainly the result of increases in the number of alveoli during this time. The period of lung development is summarised in *Figure 1-4.*

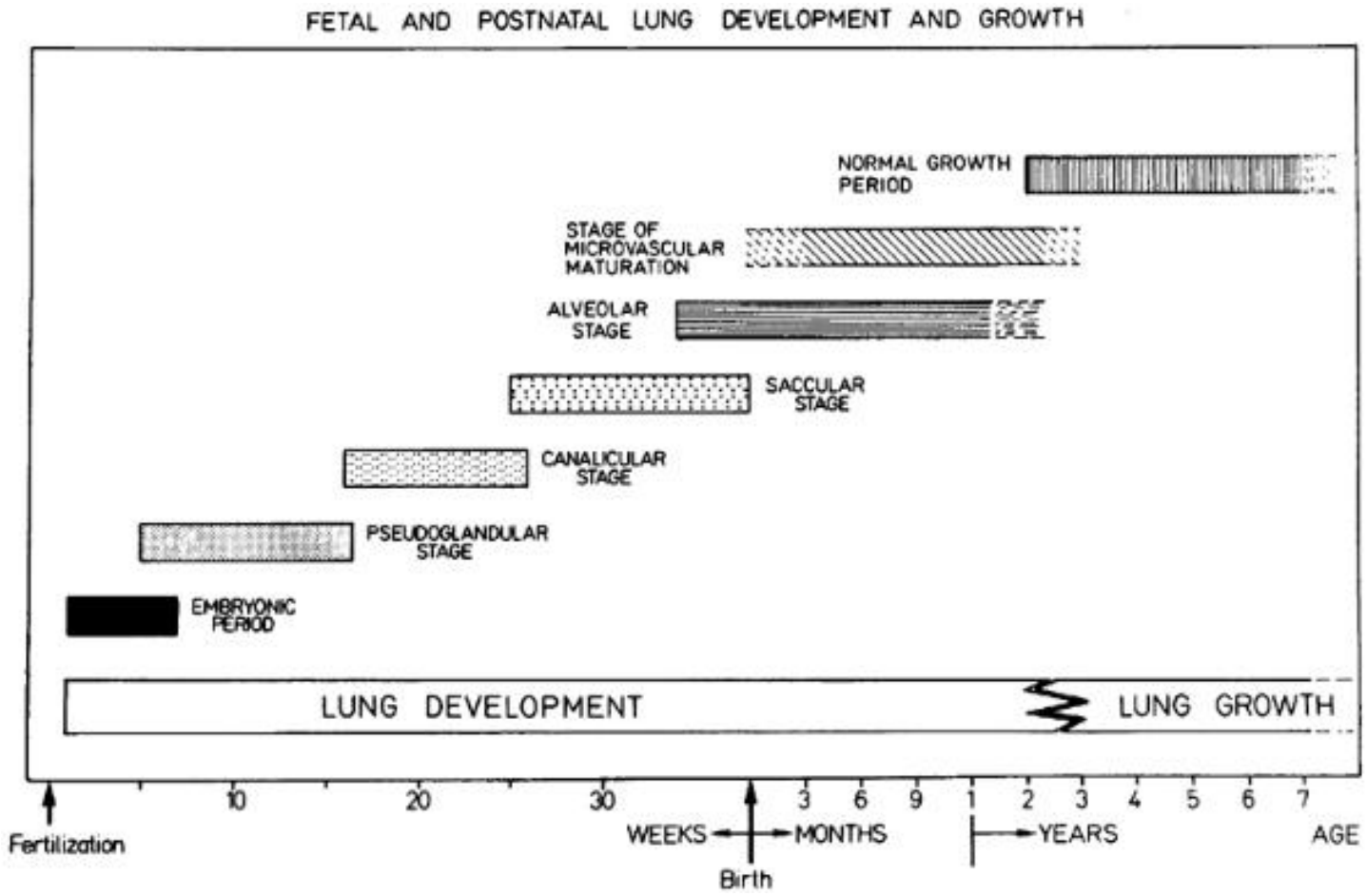

Figure 1-4 Stages and timing of human lung development.

Open-ended bars indicate that the exact start and end stages are still unknown. From Zeltner & Burri (1987).

1.2 Alveolar anatomy

1.2.1 Alveolar epithelial type I

The alveolar epithelial type I (ATI) cells are squamous epithelial cells that cover more than 95% of the surface area of the lung (Mutlu & Sznajder, 2005). The cells are highly flattened, and this morphological feature is important since it implies that the cells provide a significant barrier to the diffusion of gases (Junqueira *et al.*, 1998). Apart from gaseous exchange, the cells are now known to express aquaporin-5 (AQP5), a water channel, and at least two types of sodium-permeable ion channels (these include epithelium sodium channels; ENaC and cyclic nucleotide-gated channels; CNG) on the apical membrane (Dobbs *et al.*, 1998, Borok *et al.*, 2002; Johnson *et al.*, 2006) hence suggesting that the type I cells may contribute to another role; in the transport of ions and water.

While expressing these channels, the other functional purpose of ATI cells is to assist in the efficient diffusion of gas into and out of the blood supply. However, this characteristic leads to a major obstacle in the study of this cell type as they are extremely fragile and the isolation necessary to create cultured ATI cells is very difficult to achieve. Only with recent advancements in techniques for the isolation of ATI cells and the discovery of the epithelial Na^+ channel (ENaC) has it been possible to change the original view that ATII cells were the main source of Na^+ transport that drives the absorption of fluid; which also suggests that ATI cells play a vital role in ion transport (Johnson *et al.*, 2002; Bourke *et al.*, 2005; Helms *et al.*, 2006; Wilkinson *et al.*, 2011).

1.2.2 Alveolar epithelial type II

Type II alveolar cells are cuboidal epithelial cells found in the alveolar surface. They are characterised by the unique ability to synthesise and secrete surfactant protein and by the distinct of secretory granules called lamellar bodies. The surfactant contains a surface-active lipoprotein complex (phospholipoprotein), comprised of both a hydrophilic and a hydrophobic region. It was found in the late 1920s by von Neergaard, who identified the pulmonary surfactant by increasing the compliance of the lungs by reducing surface tension; the importance of having low surface tension is crucial in lungs of newborn infants (von Neergaard, 1929). However, the significance of his discovery was not properly understood at that time. Later in 1950s, Pattle and Clements rediscovered the importance of surfactant and low surface tension in the lungs. It was then that the discovery of the cause of respiratory distress syndrome (RDS) through the lack of the surfactant protein was determined. In early 1954, Macklin had postulated that the surfactant is secreted by alveolar type II cells (Macklin, 1954) and later in 1977, Mason and Williams developed the concept of alveolar epithelial type II cells as a defender of the alveolus. It was established that ATII cells synthesise and secrete surface-active material (Mason & Williams, 1977). Taken together, these findings suggest that the surfactant lipid produced by ATII is important for reducing surface tension, and preventing collapse of the alveolus. The immaturity of the cells is a major contributing factor to RDS in premature infants (O'Brodovich, 1996). Thus many studies have used this type of cell as it has become much easier to isolate and culture since Kikkawa and Yoneda first proposed a method of isolation for *in vitro* studies in 1974 (Kikkawa & Yoneda, 1974).

Since then, ATII were thought to be the primary source of Na^+ transport in the alveoli and various studies have shown that this type of cell contains ENaC subunits using Northern Blot Analyses, RT-PCR, immunoprecipitation and *in situ* hybridisation (Yue *et al.*, 1995; Farman *et*

al., 1997; Talbot *et al.*, 1999). Although it was originally thought that the ATII cells were the only side of active Na^+ transport in the alveolar region, studies using the isolated ATI cells and lung slice preparations have shown that these cells are also capable of active Na^+ transport (Johnson *et al.*, 2002; Bourke *et al.*, 2005; Helms *et al.*, 2006; Eaton *et al.*, 2006; Wilkinson *et al.*, 2011). ATII and ATI cells may thus contribute to the removal of liquid from the alveolar region, more recent work suggests that the type I cells might also contribute to this process (Wilkinson *et al.*, 2011). It is therefore clear that both alveolar type I and type II cells can express the transport proteins needed to allow a paradigm for fluid absorption, in which the uptake of Na^+ generates the osmotic gradients that fluid then follows passively. It was first thought that the bulk of Na^+ transport occurred via ENaC in the ATII cells, while ATI provided a route for water movement via the aquaporin-5 channel (Nielson *et al.*, 1997). However, due to the large surface area of ATI cells, it seemed likely that they too, might play a significant role in fluid absorption.

1.2.3 Functions of the blood-air barrier of the alveolus

In the alveolus, gases, liquids and solutes (e.g., metabolites) are exchanged or transported between the blood and air compartments. Moreover, the surfactant and exogenously presented material is taken up for degradation or inactivation into various cells. The trafficking molecules have to travel through different extracellular and cellular compartments by passive and sometimes also active processes. Therefore, there is a need for both ATI and ATII cells to be organised in the blood / gas barrier. Concomitant with the development of various lung structures is the cellular differentiation of ATI and ATII cells occurring as the alveolar epithelium matures. Therefore, during the first four months of gestation the epithelial lining is more or less columnar to cuboidal (Boyden, 1977; Mason & Williams, 1977; Fehrenbach, 2001). By six months, ATI and ATII cells can be distinguished in the more localised and differentiated zones of pseudo-cuboidal cells.

Since ATI cells contain an abundance of caveolae, they are thought to be involved in various transport processes. A growing body of evidence suggests that caveolae are more stable structures than coated pits, and that they also pinch off from the plasma membrane and form small vesicles in the cytoplasm (McElroy *et al.*, 2004; Barth *et al.*, 2009; Wang *et al.*, 2011; Maniatis *et al.*, 2012). Furthermore, several receptors are expressed/congregate in the caveolae, which might act as the centre for control of the activity of receptors and molecules involved in cell signalling. Insights into the importance of caveolin-1 for the function of the distal lung blood–air barrier have mainly emerged from knockout mice studies, where the loss of caveolin-1 resulted in severe lung abnormalities and thickened alveolar septa (Razani *et al.*, 2001). Under pathophysiological conditions, the alveolar epithelial cell damage is associated with lower levels of caveolin-1, which is probably mainly due to the high vulnerability of ATI cells and consequently the significant loss of ATI cells in response to several sources of injury (Kasper *et al.*, 1998).

ATII cells are identified by their granular and cuboidal appearance, which is a result of the dense packing of cytoplasmic organelles (indicating metabolically active cells) and lamellar bodies, which are dense and layered organelles that synthesise and store pulmonary surfactants (Boyden, 1977; Mason & Williams, 1977; Crapo *et al.*, 1980; Fehrenbach, 2001). The major function of a surfactant, which is a mixture of proteins and the lipid disaturated dipalmitoyl phosphatidylcholine, is to reduce the surface tension, thus facilitating lung expansion during inhalation. Although ATII cells are small in diameter (~400 μm^3 in rats and ~900 μm^3 in humans), they are still essential for proper gas exchange. Situated at the corners of the alveolar sacs, ATII cells represent little obstruction to gaseous diffusion and are fed by a capillary network. Haddard (2002) in his review further added that these cells are intracellularly furnished with cytoplasmic organelles associated with the biosynthesis of surfactant

phospholipid and related proteins. The characteristics are to serve ATII cells as thin, gas-permeable entities for diffusion and act as a protective barrier against water and electrolyte leakage (Boyden, 1977; Mason & Williams, 1977; Fehrenbach, 2001).

1.3 Fluid transport processes within the lung

1.3.1 Fluid secretion

It has long been known that the lung develops as a fluid filled organ, and it is also clear that this liquid must be removed from the airspaces by the time of birth since the retention of even a small volume of liquid in the alveolar space will provide a significant barrier to gas exchange. This view derived initially from Potter & Bohlender, (1941) who noted that the lung tissues distal to congenital airway obstructions became distended with liquid, implying that liquid must be produced within the lung tissues themselves. This view is concurrent with that of Jost & Policard (1948), who were studying a ligated foetal rabbit trachea and attempting to induce hypopituitarism, when they noted that the lung tissue distal to this artificial obstruction became distended with liquid. The most compelling explanation for foetal lung liquid was simply inhaled amniotic fluid, based on the analysis of the composition of foetal lung liquid and amniotic fluid. These analyses showed that lung liquid contained higher levels of Na^+ (150 mM vs 113 mM) and Cl^- (157 mM vs 87 mM) but lower concentrations of protein (27 μgml^{-1} vs 100 μgml^{-1}) and bicarbonate (2.8 mM vs 19 mM) which disproved the notion that the lung liquid was simply inhaled amniotic fluid (Adams *et al.*, 1963; Adamson *et al.*, 1969). Furthermore, parallel analyses showed that foetal lung liquid also differed from plasma implying that it must be formed by an active secretory process rather than as a plasma ultra filtrate (the maintenance of the solute concentration differences require that the lung epithelium be a barrier to diffusion). This view is supported via radioisotope flux studies, which indicated that Cl^- was being actively secreted into the lung lumen whilst Na^+ followed by passive electro-diffusion (Olver & Strang, 1974). Subsequent studies also showed that changes in vascular filtration

pressure had no effect upon the rate at which this liquid was produced (Carlton *et al.*, 1992 a,b). The prerequisite for net secretion is dependent on the impermeable and the experiments using water soluble non-electrolytes confirmed that the foetal lung epithelium is 'tight'. In terms of pore theory, the permeability pathways can be characterised as cylindrical pores of 0.6 nm radius, compared to pores of 15 nm in the pulmonary endothelium (Normand *et al.*, 1971).

The mechanism proposed by earlier observations has been further investigated by subsequent studies of a model in several different vertebrate epithelia, including the elasmobranch rectal gland, the avian salt gland (Silva *et al.*, 1977) and in the canine tracheal epithelium (Welsh *et al.*, 1980). These tissues, in common with the secretory epithelia of the foetal lung, actively secrete Cl^- into the lumen, and Cl^- influx across the basolateral membrane of the secretory cells is mediated via an electroneutral Na^+-K^+-$2Cl^-$ co transport system which utilises the inwardly directed electrochemical Na^+ gradient, maintained by the Na^+-K^+-ATPase, as a source of potential energy. Confirmation of the Cl^- influx mechanism in the lung has come from several studies of sheep and guinea pigs (Cassin *et al.*, 1986; Thom & Perks, 1990). This secondary active ion transport process can thus raise intracellular Cl^- above the electrochemical equilibrium and so establish an outwardly directed, electrochemical Cl^- gradient. Cl^- can therefore enter the lumen via apically located anion channels and, since the efflux process is electrogenic, this establishes an electric driving force for the passive movement of Na^+. Together, the movement of these ions provide an osmotic gradient favouring water movement into the lung lumen. This model was subsequently shown to provide an almost universal mechanism that allowed vertebrate epithelia to secrete fluid.

1.3.2 Fluid reabsorption

Whilst the secretion of liquid into the lumen of the developing lung is clearly important for the proper growth and differentiation of the lung tissues, it is imperative that this liquid is removed from the potential airspaces at birth since the presence of even a small volume of liquid can impair the proper oxygenation of the blood. Indeed, the retention of liquid in the lung, together with impaired surfactant secretion, are characteristic features of neonatal Respiratory Distress Syndrome (RDS), the most common cause of death amongst newborn and premature infants in the developed world. The first indication that birth is associated with significant changes to lung fluid balance came from studies conducted by Faure-Fremiet & Dragiou (1923), who simply measured the water content of lungs obtained from new-born lambs. The lungs of the newborn lambs had a water content of 76%-78%, which was lower than foetal tissue (87%-89%). About 4 decades later, a detailed study was conducted in a similar way, but with rabbit lungs (Aherne & Dawkins, 1964). Both studies confirmed that birth is associated with a fall in lung water content and the data suggested that this process begins immediately after birth and is completed within two hours.

The removal of lung liquid was believed to be an entirely passive process that was dependent upon the compression of the chest during birth. However, several studies indicate that this is not the case as births by caesarean section do not impair the removal of liquid from the lung (Bland *et al.*, 1980 a, b). Moreover, a physical occlusion of the trachea does not prevent the removal of liquid from the lungs of experimental animals (Berger *et al.*, 1998; Brown *et al.*, 1983). The lung starts to reabsorb fluid during labour, and this is first mediated via adrenaline (Lagercrantz & Bistoletti, 1977), indicating a possible role for this hormone as the trigger for fluid clearance. Subsequent experiments conducted by Brown and colleagues showed that sensitivity to adrenaline increased with gestational age in foetal lambs, which caused a slowing of liquid secretion (before 130 days) turning to liquid absorption after 147 days (Brown *et al.*,

1983). This observation was confirmed by Olver *et al.*, (1986) through ionic flux. Using similar methods to Olver & Strang, (1974), they found that Na^+ is actively transported from the lung lumen to the plasma. Furthermore, they found that Na^+ transport could be blocked by amiloride, which also prevented adrenaline induced liquid absorption (Olver *et al.*, 1986). Therefore, the data suggested that Na^+ flux could be the determining factor in driving liquid absorption. They also proposed that Na^+ and Cl^- transport were connected and that Cl^- transport was a result of co-transport with Na^+ driven by the Na^+-K^+-ATPase via, at the time, an unidentified transporter in the basolateral membrane. The opening of apical Na^+ channels would lead to Na^+ absorption also driven by Na^+-K^+-ATPase, which would cause the uptake of fluid due to a change in osmotic gradients. In the early nineties, a study investigating the administration of amiloride to the trachea of newborn guinea pigs revealed a phenotype in the animals similar to RDS (O'Brodovich *et al.*, 1990). This study also provided evidence indicating that Na^+ absorption was mediated through an apical Na^+ channel and therefore was thought to play a role in clearing the lung fluid. The effect of adrenaline also appears to be mediated via cyclic adenosine monophosphate (cAMP), as administration of cAMP to the foetal lambs' lung mimics its effects and the response can be blocked by amiloride (Walters *et al.*, 1990). This indicates that this mechanism involves epithelial sodium channels (ENaC). However, Na^+ transport can also be assisted by other cation channels; cyclic nucleotide-gated channels (CNG) and perhaps nonselective cation channels (NSCC).

1.3.3 The ionic transport in fluid secretion and absorption

The hydration of the lung is very important for optimal function, therefore water movement across a selective permeable membrane of cells must be monitored through osmosis, from an area of high water concentration (or low solute concentration) to an area of low water concentration (or high solute concentration). Proteins that span the plasma membrane form

channels that allow for the selective transport of ions. The active transport of specific ions leads to the generation of an osmotic gradient that either favours fluid secretion or absorption.

Prior to the maintenance of lung hydration through fluid secretion, Cl^- enters the cell via the basolateral Na^+-K^+-$2Cl^-$ co-transporter (NKCC) thereby elevating the intracellular Cl^- concentration, allowing Cl^- to exit the apical Cl- channels (*Figure 1-5i*). The apical entry for Cl^- can be mediated by few channels; the cystic fibrosis transmembrane conductance regulator (CFTR), Ca^{2+} activated Cl^- channels and voltage or volume regulated (CIC family) Cl^- channels (see review, Anderson *et al.*, 1992). The action of secretion generates a negative potential in the lung cells and thus creates a driving force for Na^+ to be passively transported into the cells to compensate for the difference and maintain a stable gradient. Whilst the osmotic gradient is established, water flows down through the cells towards the apical cells to provide hydration. This was first suggested by Silva *et al.*, (1977) as a form of secondary active transport.

This secretory characteristic of the foetal lung epithelium becomes absorptive through activation of Na^+ channels in the apical membrane at birth and is sustained into adult life (*Figure 1-5ii*). The maintenance of a thin liquid film is important for gas exchange, since the presence of even a small volume of fluid will provide a significant barrier to the diffusion of gases. This can be done through an active transport of Na^+ which will then generate an osmotic gradient to favour fluid re-absorption. The secondary active transport would be through the Na^+/K^+-ATPase exchanger in the basolateral membrane, to pump out Na^+ at a ratio of three Na^+ ions out for two K^+ ions in. This helps to keep the intracellular concentration of Na^+ low (~10 mM), therefore creating an electrochemical gradient that favours the apical entry of Na^+ from the surrounding fluid in the apical membrane. The ratio of Na^+ out to K^+ ions in means that there is a net loss of positive charge from the cell, resulting in a negative potential; thus the effect of the Na^+/K^+-ATPase is to generate an electrochemical gradient that favours Na^+ entry

into the cell, for example via ENaC. In order to prevent depolarisation of the cell from the build up of K^+, K^+ channels present in the basolateral membrane will allow K^+ to "leak-out" out of the cell down the chemical gradient. The polarised nature of the respiratory epithelium means that vectorial transport of ions can be achieved.

The regulation of Na^+ absorption and Cl^- secretion and the balance between these is the major determinate of respiratory hydration and therefore the mechanism involved in their regulation is of critical importance. Furthermore, the identification of the specific channels involved is of equal importance so that strategies can be developed to treat diseases which result from impairment of these processes.

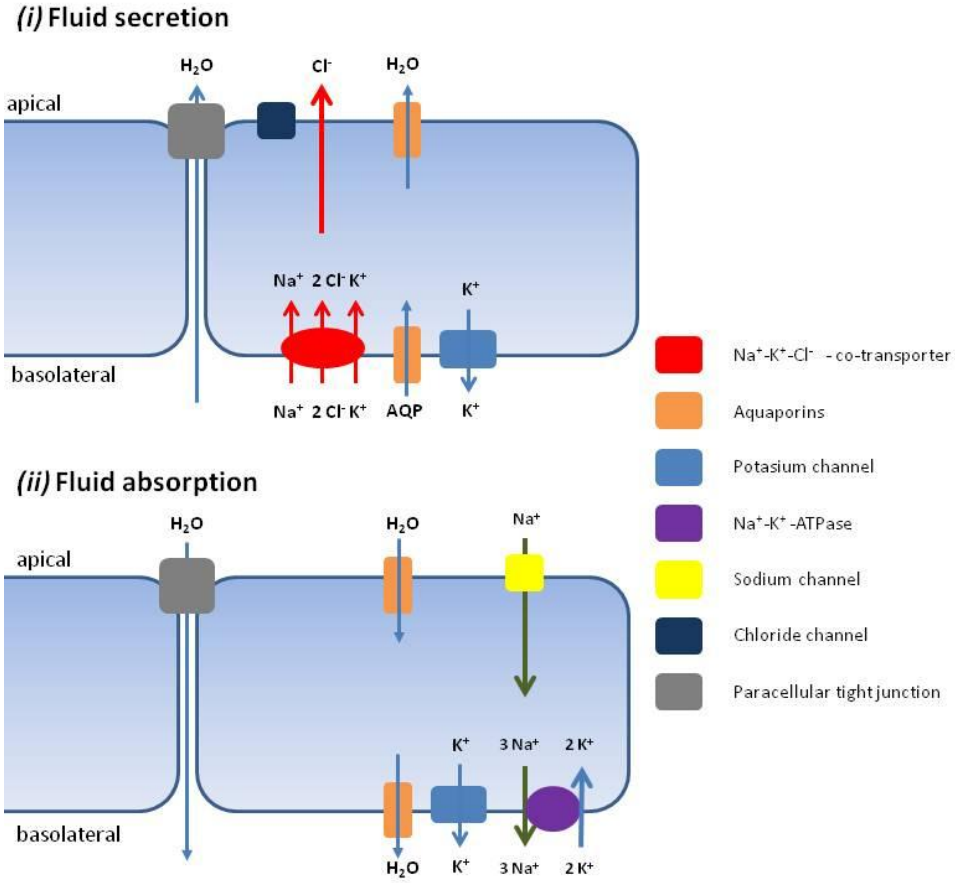

Figure 1-5: Schematic diagram of lung fluid secretion and absorption

(i) Chloride ions (Cl^-) enter the cells via the Na^+-K^+-Cl^- channel co-transporter, thus raising the intracellular [Cl^-] and Cl^- is secreted through apical Cl^- channels. This allows Na^+ transport into the cells and creates an osmotic gradient that favours the secretion of fluid through the paracellular pathway or possibly through aquaporins (AQP). K^+ channels help to prevent depolarisation.

(ii) Na^+-K^+-ATPase pumps out 3 Na^+ from the basolateral membrane (whereas only 2 K^+ is pumped in), to create a loss of positive charge in the cells which favours Na^+ entry via apical Na^+ channels, thus creating an osmotic gradient favouring fluid absorption via the paracellular pathway or via AQP located in the membrane. K^+ exits the cell via the basolateral K^+ channels to prevent depolarisation from K^+ build up.

1.3.4 The hormonal regulation of lung fluid absorption

Walters & Olver, (1978) showed that the infusion of adrenaline or isoprenaline slowed the rate of lung fluid secretion in mature foetal sheep. This concurs with the study performed by Lagercrantz and Bistoletti (1977) who showed that the concentration of adrenaline in the foetal plasma rose to very high concentrations during labour and birth. The reabsorption of lung fluid occurs during the last few hours before birth (Brown *et al.*, 1983). As gestation advances, these hormones inhibit fluid secretion until labour, when the direction is reversed to induce fluid absorption (Walters & Olver, 1978). The authors also suggested that the switch could be induced by β-adrenoceptors and mimicked by a cell-permeant cAMP analogue (Walters & Olver, 1978; Olver *et al.*, 1986; Walters *et al.*, 1990). Moreover, adrenaline and cAMP-evoked liquid absorption are both inhibited by amiloride (Olver *et al.*, 1986; Walters *et al.*, 1990), a Na^+ channel antagonist that also abolishes the natural occurrence of lung liquid seen during labour and birth (O'Brodovich, *et al.*, 1990). As β-adrenoceptor antagonists also inhibit this process (Brown *et al.*, 1983), this body of work indicates that adrenaline-evoked Na^+ transport underlies the absorption of lung liquid seen at birth. Subsequent studies suggested that this effect could be mimicked by intratracheal administration of dibutyryl cyclic adenosine monophosphate (db-cAMP) (Olver *et al.*, 1987; Walters *et al.*, 1990), thyroid hormones of triiodothyronine (T_3) (Barker *et al.*, 1990), and hydrocortisone (Barker *et al.*, 1990, 1991).

1.3.5 The role of glucocorticoids and thyroid hormone

The increased level of adrenaline is preceded by a rise in circulating glucocorticoids (GC) and thyroid hormone before labour (Baines *et al.*, 2000) suggesting that both hormones are involved in the maturation of the Na^+- absorptive phenotype to allow adrenaline to stimulate Na^+ absorption. This was demonstrated by the lack of thyroid in an immature foetal sheep which prevented a normal response to adrenaline (Barker *et al.*, 1988). The authors also suggested that

adrenaline is dependent on a component downstream of cAMP, where a thyrodectomy has reduced the response to both adrenaline and synthetic cAMP (db-cAMP) (Barker *et al.*, 1988). A subsequent study (*Figure 1-6*) also confirmed that such absorptive features in the thyrodectomised immature foetus produced a better prognosis by clearing more water from the lung when infused simultaneously with both thyroid and glucocorticoid hormones (Barker *et al.*, 1991).

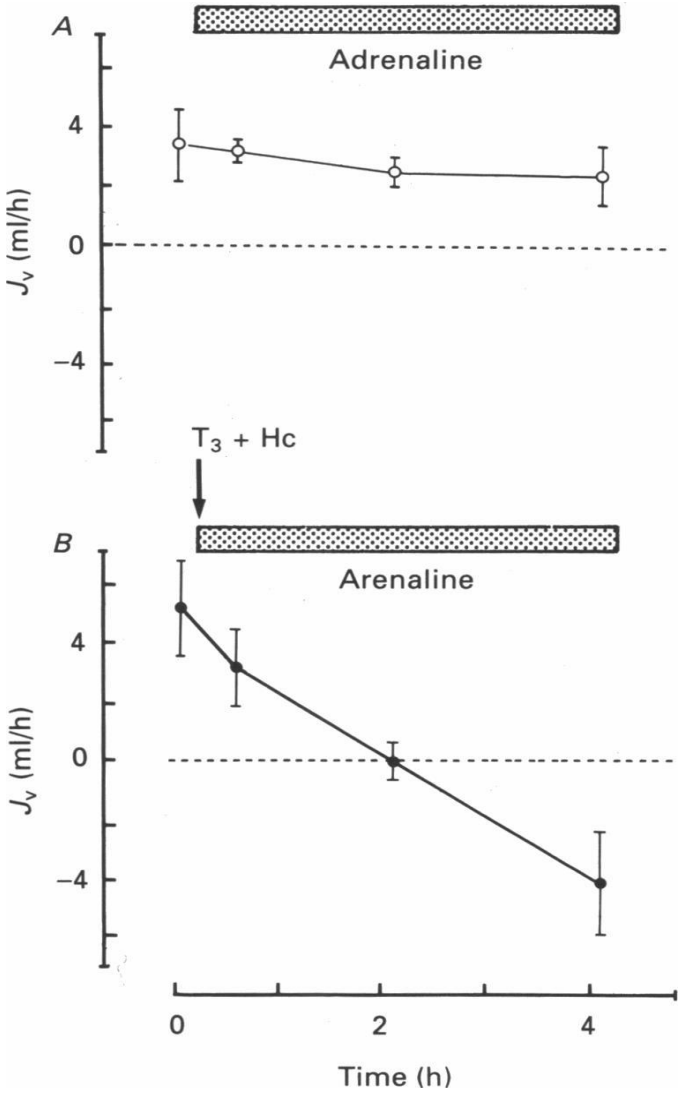

Figure 1-6: Effects of thyroid (T_3) and glucocorticoid (HC, hydrocortisone) hormones on the thyroidectomised foetal sheep (Barker *et al.*, 1991).
(A) A thyroidectomised foetal sheep failed to respond to adrenaline whilst *(B)* the absorption rate increased (- J_v, *i.e.*, fluid clearance from the lung) when infused with both T_3 and hydrocortisone (Hc).

Later on, subsequent studies of rat foetal distal lung epithelial (FDLE) cells also showed that the β-adrenoceptor isoproterenol did not evoke an increase in Na^+ transport when applied to cells that had been maintained in a fully defined medium that was devoid of hormones (Ramminger *et al.,* 2002). Since this result contrasts with several earlier studies of cells maintained in hormone-supplemented medium (Barker *et al.,* 1993; Barquin *et al.,* 1997; Baines *et al.*, 2000), the data illustrate that the action of β-adrenoceptor agonists is dependent on prior exposure to other hormones. Since earlier studies of foetal lambs had suggested that the adrenaline-evoked removal of liquid from the lung was dependent upon prior exposure to thyroid / GC hormones, subsequent experiments explored the effects of exposing them to T_3 and dexamethasone (thyroid and GC hormone respectively), either alone or in combination. Although exposure to the individual hormones did not restore sensitivity to the β-adrenoceptor agonist of isoproterenol, clear responses were seen in cells that had been exposed to both hormones (Collett *et al.,* 2002). Experiments undertaken *in vivo* and *in vitro* therefore demonstrate that adrenaline-induced Na transport is dependent on prior exposure to T_3 / GCs (Ramminger *et al.*, 2000; Ramminger *et al.,* 2002). Taken together, these data demonstrate the importance of both hormones for the maturation of lung, as they work synergistically allowing additional treatment with adrenaline to stimulate the response of Na^+ and to clear the lung fluid. Such stimulation is recognised through the sensitivity to amiloride (Olver *et al.,* 2004).

1.4 The epithelial sodium channel (ENaC)

One of the molecular candidates that affects Na^+ absorption in the lung is the epithelial Na^+ channel (ENaC). The idea that ENaC was critical for liquid clearance came from Hummler's studies of α-ENaC knock-out mice. These animals had water logged lungs and were therefore unable to survive for more than ~48 h. It was subsequently shown that the expression of a rat α-ENaC transgene rescued this phenotype. These mice did however, have abnormally low rates of sodium absorption. Although they did not display any physiological abnormalities under standard conditions, they were abnormally susceptible to pulmonary oedema (Hummler *et al.*, 1996). These studies are particularly important because they provide a link between the studies of foetal lambs undertaken by Olver & Walters, (1979), Barker *et al.,* (1983) and later studies of ENaC in different cell types.

1.4.1 Structure of ENaC

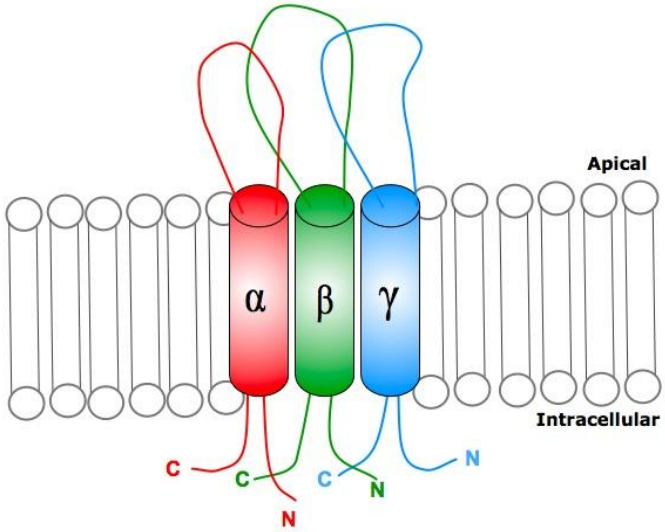

Figure 1-7 The classical epithelium sodium channel (ENaC)

ENaC is composed of three subunits: α-, β-, and γ- which each have an intracellular carboxy (C) and amino (N) terminal.

The ENaCs are composed of three homologous subunits (*Figure 1-7*), called alpha (α), beta (β) and gamma (γ); they consist of intracellular amino and carboxyl termini and two membrane spanning domains connected by large extracellular loops (Carattino *et al.*, 2006; Bruns *et al.*, 2007; Hughey *et al.*, 2004b). These subunits are structurally homologous, and their amino acid sequences display ~30% similarity (Canessa *et al.*, 1994). However, the preliminary study identified that the ENaC α-subunit regions in the *Xenopus laevis* oocyte expression system produce amiloride sensitive Na^+ currents (Canessa *et al.*, 1993). Whilst this is similar in a heterologous system, these currents were not noticed for either β- or γ-ENaC (Canessa *et al.*, 1994). Studies of channel subunits expressed in *Xenopus* oocytes suggest that the independent expression of α-ENaC, but not β or γ-ENaC, is associated with an amiloride sensitive Na^+ current. However, the co-expression of all three subunits increases the magnitude of this current ~100 fold, a result which suggests that all three subunits are needed to form functional channels. It would therefore appear that fully functional ENaC is composed of three separate subunits, α-ENaC being the major pore forming subunit, while β-ENaC and γ-ENaC subunits confer selectivity.

The functionality of the multimeric protein can be determined by observing the interaction of the subunits in a rat's ENaC (Canessa *et al.*, 1994); followed by the identification of channel co-expression with each subunit (McDonald *et al.*, 1995; Bruns *et al.*, 2003). Taken together, the data from previous studies proposed that α-ENaC can generate a small current on its own but potentiates a maximum current when expressed with β- and γ-ENaC (Canessa *et al.*, 1994; McDonald *et al.*, 1995; Awayda *et al.*, 1997; Bruns *et al.*, 2003). Therefore, it is thought that all subunits are required for the development of the fully functional form of ENaC. While the stoichiometry of the subunits is not clear, it is generally thought be a heterotrimer, 1α : 1β : 1γ. This is structurally similar to that determined from the crystal structure of the acid-sensing ion

channel 1 (ASIC1) in the chicken (Jasti *et al.*, 2007). However, other stoichiometries have been proposed, such as 2α : 1β : 1γ (Firsov *et al.*, 1998, Kosari *et al.*, 1998, Anantharam & Palmer, 2007) and the nonameric structure of 3α : 3β : 3γ (Snyder *et al.*, 1998; Eskandari *et al.*, 1999; Staruschenko *et al.*, 2005).

A molecular basis for the interaction of subunits was first suggested after observing their presence at the surface of the apical membrane (Firsov *et al.*, 1996). Where the three subunits are structurally similar, they are thought to form a pore (Schild *et al.*, 1997; Benos & Stanton, 1999). The subunits are assembled in the endoplasmic reticulum, post-translationally modified in the Golgi network and then trafficked to the plasma membrane together (Cheng *et al.*, 1998; Fyfe *et al.*, 1998; Horisberger, 1998). However, the channels formed by the co-expression of α- and γ-ENaC in the absence of the β-subunit have better channel activity because they stimulate more Na^+ transport and are more sensitive to amiloride than those formed by the co-expression of α- and β-ENaC which display a lower sensitivity to amiloride (McNicholas & Canessa, 1997). The finding also suggests that all three subunits contribute to the channel pore, indicating that α-ENaC cannot form the pore by itself, but that the other subunits serve as chaperones to transport and stabilise it in the membrane (Canessa *et al.*, 1994; Waldmann *et al.*, 1995; Prince & Welsh, 1998). However, there is a possibility that native cells contain a variety of different channel types with different stoichiometries (McNicholas & Canessa, 1997) which could account for the variability of properties of amiloride-sensitive channels found in different tissues (Smith & Benos, 1991; Palmer, 1992).

The ENaC protein consists of an N- and a C-terminus, and together these play a role in protein function at the cell surface. The cytoplasmic N-terminus of ENaC is thought to be involved in the assembly of the subunits (Benos & Stanton, 1999) and endocytic retrieval (Gründer *et al.*, 1997; Horisberger, 1998; Prince & Welsh, 1998) which involves lysine residues (Staub *et al.*,

1997a; Goulet *et al.*, 1998). The hydrophobic region of the N-terminus is also thought to form part of the channel pore (Canessa *et al.*, 1995) in addition to playing a role in channel gating (P_o) (Grunder *et al.*, 1997, Berdiev *et al.*, 1998). The N-terminus in α-ENaC is required for maximal activity whilst β- and γ-ENaC play an important role in determining the half-life of the channel (Staub *et al.*, 1997a). In addition to this, amino acids in each protein subunit in the extracellular domain distinguish the functionality of ENaC. This ensures the utility of the domain where each subunit can interact with the extracellular solution. This involves glycosylation (Rotin *et al.*, 2001) and amiloride binding (Benos, 1982) which may exist in the α-subunit (Berdiev *et al.*, 1998) but not in both β- and γ-ENaC subunits (Schild *et al.*, 1997).

The C-terminal transmembrane domain appears to face the extracellular serine-proteases that lead to ENaC activation. This is associated with a linker region that lies in a catalytic domain that allows ENaC interaction with other proteins at the cell surface (Hooper *et al.*, 2001). This domain is involved in the protease cleavage that potentiates an amiloride sensitive transepithelial current in the epithelial cells of kidney (Kleyman *et al.*, 2006; Sheng *et al.*, 2006) and lung (Planés *et al.*, 2005; O'Brodovich *et al.*, 2008; Mace & Baines, 2009). The cleavage initiates full activation of ENaC, especially in both α- and γ-ENaC (Knight *et al.*, 2008). This region also contains an internalisation sequence which is rich in proline motifs (PY-motif). A mutation or deletion of the PY-motif can result in increased ENaC activity which can lead to Liddle's Syndrome, an inherited form of hypertension.

1.4.2 ENaC expression in the lungs

ENaC mediates a rate-limiting step in transepithelial sodium absorption in airway epithelial cells. In the developing rat foetal lung, all mRNA subunits of ENaC are expressed even before birth when there is no Na^+ absorption. However, the proteins are expressed after birth which coincides with the phenotype switches that trigger reabsorption of the liquid from the alveolar

lumen (Tchepichev *et al.*, 1995; Watanabe *et al.*, 1998; Talbot *et al.*, 1999). Kellenberger and Schild, (2002) suggested that liquid clearance might correspond to the expression of α- and γ-ENaC in the distal foetal mouse lung. Similarly, Talbot *et al.*, (1999) and Smith *et al.*, (2000) revealed an increment in these subunits in late foetal and early post-natal life, when the lung turns from a secretory to an absorptive condition. However, recently Beard and colleagues found that by using sucrose gradient separation they could observe ENaC protein trafficking to the membrane. With this they have proposed that only α- and β-ENaC subunits are trafficked towards the cell membrane to allow fluid absorption (Beard *et al.*, 2011). The discrepancy between the expression of α-β-ENaC and α-γ-ENaC still needs to be elucidated, however it appears that α-ENaC plays the most important role in activating ENaC. The importance of α-ENaC was demonstrated by the neonatal death of mice that lacked an α-subunit, which impaired the sodium transport-mediated lung liquid clearance at birth (Hummler *et al.*, 1996). The heterologous expression of the α-ENaC transgene can rescue this lethal phenotype; however, it only allows a low rate of Na^+ transport and corresponds to the prognosis of oedema (Egli *et al.*, 2004). These two significant findings, together with those of other subsequent studies, have indicated the importance of the α-subunit in the clearance of fluid in the distal lung epithelium (Egli *et al.*, 2004; Helve *et al.*, 2004; Li & Folkesson, 2006; Janér *et al.*, 2010; Deng *et al.*, 2011).

Low expression of the β-ENaC subunit has also been shown to impair lung fluid clearance in the mouse (Randrianarison *et al.*, 2007). However, the lack of this subunit results in α- and γ-ENaC adopting a compensatory role to maintain ENaC activity. The importance of β-ENaC has been extensively studied by Mall and colleagues who have reported that the overexpression of the β-subunit augments sodium absorption *in vivo* (Mall *et al.*, 2004; Zhou *et al.*, 2008; Mall *et al.*, 2010; Zhou *et al.*, 2011). The over-expression of the β-subunit of ENaC in the lung cells of mice is notably similar to the clinical and pathological description of early cystic fibrosis lung

disease (Mall *et al.*, 2004; Zhou *et al.*, 2008; Mall *et al.*, 2010; Zhou *et al.*, 2011), and the lack of this subunit contributes to the clinical phenotype of pseudohypoaldesteronism (PHA) (Thomas *et al.*, 2002). However, such β-subunit expression did not affect foetal survival, in contrast to the lack of expression of α-ENaC.

The relative importance of γ-ENaC in neonatal lung fluid clearance can be demonstrated by generating mice with a γ-subunit knock-out (Barker *et al.*, 1998). Newborn mice lacking γ-ENaC have been shown to clear the fluid more slowly than control mates, suggesting that it only facilitates the clearance. It is thought that γ-ENaC is critical for renal Na^+ and K^+ transport but not transport in the lung (Barker *et al.*, 1998). When the γ-ENaC gene is mutated it produces a pathophysiology that results in the elevation of blood pressure (hypertension) in patients with Liddle's Syndrome (Födinger *et al.*, 1998; Gao *et al.*, 2001). It also contributes to the clinical phenotype of cystic fibrosis by decreasing ENaC activity (Viel *et al.*, 2008).

1.5 Factors in ENaC regulation

Many studies have been conducted on the effects of β-adrenergic agonists, oxygen, GCs and thyroid hormones that are known to contribute to the switch from fluid secretion to fluid absorption at birth, a process that requires the developmental regulation of membrane transport proteins responsible for transepithelial Na^+ transport (Sayegh *et al.*, 1999; O'Brodovich, 2004; Olver *et al.*, 2004). These are found to be intercalated to stimulate Na^+ absorption by increasing Na^+ pump activity and apical Na^+ conductance (G_{Na+}) to bring about the switch from net secretion to net absorption as lung liquid is cleared at birth. The mechanism by which ENaC can be regulated by other known factors will be described in subsequent sections.

1.5.1 Serum and Glucocorticoid-regulated Kinase (SGK)

SGK is a novel serine/threonine kinase protein that was originally cloned from a rat mammary tumour cell line (Webster *et al.*, 1993). It was first identified as a gene activated in a screen for hepatocellular gene regulation in a response to cellular hydration or cell swelling (Waldegger *et al.*, 1997). Cellular hydration is a catabolic signal, stimulating glycogenolysis and proteolysis, and inhibiting protein and glycogen synthesis. This kinase has been shown to be important in activating certain potassium (e.g., Yoo *et al.*, 2003), sodium (e.g., Chen *et al.*, 1999), and chloride channels (e.g., Embark *et al.*, 2004). The protein kinase belongs to the Protein Kinase A/G/C (AGC) family and can be activated and regulated by GCs (i.e., dexamethasone), serum and mineralocorticoids, hence the name (Webster *et al.*, 1993; Chen *et al.*, 1999; Itani *et al.*, 2002a,b). The regulation is due to the glucocorticoid response element (GRE) in the 5' flanking end of the *sgk1* gene (Itani *et al*., 2002b) and the two additional related genes; *sgk2* and *sgk3* (Kobayashi *et al.*, 1999). Whilst both SGK2 and SGK3 were thought to be responsible for ENaC (Friedrich *et al.*, 2003), they were not modulated by GCs (Kobayashi *et al.*, 1999), however SGK1 affected ENaC by increasing sodium reabsorption in *Xenopus laevis* (Chen *et al.*, 1999; Naray-Fejes-Toth *et al.*, 1999), A6 cells (Faletti *et al.*, 2002), murine cortical collecting duct cells (Helms *et al.*, 2003) and H441 lung cells (Brown *et al.*, 2008; Watt *et al.*, 2012).

1.5.1.1 Activation of SGK1

SGK1 activation is dependent on the phosphorylation of a Thr^{256} in the T-loop and a serine residue at Ser^{422}; these sites are located within the hydrophobic motif of the SGK1 protein. The activity of SGK1 has been shown to be dependent on phosphoinositide-3-kinases (PI3K) (Kobayashi & Cohen, 1999; Park *et al.*, 1999). There has been considerable debate in regard to how SGK1 is regulated. It was thought to be first phosphorylated on the hydrophobic motif by the mammalian target of the rapamycin complex 2 (mTORC2) (Garcia-Martinez & Alessi, 2008). The activation was also thought to be dependent on mTORC1 (Hong *et al.*, 2008). However, Garcia-Martinez and Alessi disputed the findings from Hong et al., (2008) by demonstrating SGK1 activity in the presence of the mTORC1 inhibitor rapamycin, this concurs with other studies (Kobayashi & Cohen, 1999; Park *et al.*, 1999). This view is further supported by a recent study, suggesting that mTORC2 is required for the phosphorylation of SGK1 at the hydrophobic motif to confer Na^+ transport (Lu *et al.*, 2010). Therefore, mTORC2 is required for the activation of SGK1 at the hydrophobic motif to promote interaction with PDK1, which results in the phosphorylation of the activation loop of SGK1 (Biondi *et al.*, 2001) and thus activity.

1.5.2 Target of rapamycin (TOR)

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that has been known to play a central role in cell metabolism, growth and proliferation. mTOR also senses cellular nutrient and energy levels and redox status (Tokunaga *et al.*, 2004). When this pathway is disrupted it can result in disease, especially cancer.

mTOR exists as two catalytic subunits of a molecular complex, mTORC1 and mTORC2; both consisting of a different associated protein. mTOR was named thus because the target of

rapamycin (TOR) was first discovered via genetic and molecular studies of rapamycin-resistant mutants of *Saccharomyces cerevisae* that identified mTORC1 and mTORC2 as the targets of rapamycin (Wedaman *et al.*, 2003). mTORC1 contains the regulatory associated protein TOR (Raptor) (Kim *et al.*, 2002) and a G-protein β subunit like protein, also known as LST8 (Kim *et al.*, 2003). The complex of mTORC1 has been recently identified as a binding partner of proline-rich PKB substrate 40 kDa (PRAS40) thus showing the role of PRAS40 as a substrate for TORC1 phosphorylation (Fonsesca *et al.*, 2007; Oshiro *et al.*, 2007). In addition, there are also other characterised targets of TORC1; p70-S6 Kinase 1 (S6K1) (Fingar *et al.*, 2004) and 4E-BP1, the eukaryotic initiation factor 4E binding protein (Hara *et al.*, 1997). Therefore, the phosphorylation of S6K1 at residue Thr^{389} is often used for the mTORC1 assay as well as PRAS40 (Yang *et al.*, 2006; McGee *et al.*, 2008; Mansley & Wilson, 2010). mTORC1 activity can be inhibited using rapamycin, which has been described as an exquisitely specific inhibitor of TORC1 (Bain *et al.*, 2007).

mTORC2 is composed of mTOR, a rapamycin-insensitive companion of mTOR (Rictor) (Sarbassov *et al.*, 2004), LST8, mammalian stress-activated protein kinase interacting protein 1 (mSin1) (Jacinto *et al.*, 2006) and proline-rich protein 5 (Woo *et al.*, 2007). mTORC2 appears to possess the activity of a previously elusive protein known as 'PDK2' by phosphorylating the serine/threonine protein kinase B (PKB) at Ser^{473} (Garcia-Martinez & Alessi, 2008) and further stimulates PKB phosphorylation at Thr^{308} by PDK1 leading to full PKB activation (Sarbassov *et al.*, 2005). mTORC2 is acutely insensitive to rapamycin, however prolonged treatment with rapamycin inhibits assembly of the complex (Sarbassov *et al.*, 2006). The recent discovery of the specific inhibitor of TOR, TORIN1 (Thoreen *et al.*, 2009; Feldman *et al.*, 2009) has given new insights into the role of mTORC2.

1.5.3 Glucocorticoids (GCs)

As the name glucocorticoid implies, it involves a role in glucose metabolism, synthesis in the adrenal cortex and their steroidal structure. GCs are required for the negative feedback mechanism in the overactive immune system, such as allergies, asthma, and autoimmune diseases. Therefore, they have a wide range of effects throughout the body including stimulation of gluconeogenesis (Christ-Crain *et al.*, 2008; Buren *et al.*, 2008), immunity (Ricardi *et al.*, 2010; Cutolo *et al.*, 2011) and lung maturation (Shanks *et al.*, 2010). One of the most widely used clinical GCs is dexamethasone, a synthetic cortisol, which is used to support the essential function of cardiovascular, metabolic, immunologic and homeostatic processes. However, the effect of dexamethasone is long-acting and its potency is about 25 times greater than that of hydrocortisone (cortisol) (Zoorob *et al.*, 1998). It is often used in the therapeutic treatment of inflammatory disease, including lung injury (Dagenais *et al.*, 2006), prevention of RDS (Liggins & Howie, 1972), paediatric asthma (Ebrahimi & Sarkari, 2007) and clearing alveolar epithelial fluid from the adult lung (Folkesson *et al.*, 2000).

Dexamethasone also has an effect on oxidative stress as shown by Xu & Chu (2007). This study demonstrated that dexamethasone could still activate α-ENaC expression in the presence of H_2O_2, which induces pulmonary oedema and acute lung injury. This finding supports the research done by Otulakowski *et al.*, (2006) which demonstrated a specific translational regulation of α-ENaC in response to physiological regulators such as steroid hormones and O_2. Otulakowski and his colleagues conducted a subsequent study investigating the integrated effects of synthetic glucocorticoid on the changes in partial pressure of oxygen (PO_2) in foetal distal lung epithelia (Otulakowski *et al.*, 2007). They concluded that dexamethasone increases the expression of α-ENaC in the apical membrane in an appropriate percentage of O_2, which corresponds to the postnatal O_2 environment. This result concurs with previous findings, which revealed that short term up-regulation of dexamethasone augments ENaC trafficking via the

serum and glucocorticoid regulated kinase (SGK-1) (Itani *et al.*, 2002a,b). Guney *et al.*, (2007) undertook studies which explored the extent to which dexamethasone was able to expose the effects of hypoxia-induced inhibition of alveolar Na^+ transport. Dexamethasone successfully induced a two-fold increase in alveolar fluid clearance in a normoxic rat, whilst decreasing reabsorption could be seen under hypoxia conditions; thus, the hormone prevented the hypoxia-induced inhibition. Furthermore, dexamethasone slightly increased the expression of α- and γ-ENaC in the whole rat lung (Otulakowski *et al.*, 2006).

GCs are involved in ENaC regulation through the redistribution of ENaC subunits from intracellular compartments to the membrane (Masilamani *et al.*, 1999; Loffing *et al.*, 2000; Frindt *et al.*, 2001; Ergonul *et al.*, 2006) particularly during the early response. Given this, GCs play an important role in increasing Na^+ transport. To achieve this process, a GC binds to a cytosolic GC receptor complex which targets steroid response elements and alters gene transcription of ENaC. This can be seen after 1 h of exposure to the hormone (Itani *et al.*, 2002a). The increment is dependent on the gene transcription and translation of ENaC subunits to produce more protein at the surface. It has been suggested that SGK1 might also be involved in this increase in ENaC gene transcription (Boyd & Náray-Fejes-Tóth, 2005) which leads to an increase in Na^+ absorption at the apical membrane. A subsequent study by Brown *et al.*, (2008), which was recently continued by Watt *et al.* (2012), has demonstrated that dexamethasone-induced airway epithelial H441 cells promote a depolarisation of the membrane potential resulting in an increase in Na^+ conductance. Such an increment is thought to be synchronised through the stimulation of transcription of ENaC subunits. However, α-ENaC contains glucocorticoid response elements (GRE) at its 5' flanking region that respond to the action of dexamethasone (Otulakowski *et al.*, 1999; Sayegh *et al.*, 1999; Itani *et al.*, 2002b; Thomas & Itani, 2004; McTavish *et al.*, 2009) which is not found in other subunits. Whilst the idea that SGK1 directly regulates α-ENaC transcription is tempting, a recent study (McTavish *et al.*,

2009) found that the α-ENaC gene promoter can be regulated independently of SGK1, although the SGK1 pathway does provide a mechanism that allows this transcriptional response to dexamethasone to be enhanced.

1.5.4 Insulin

The main physiological role of insulin is to stimulate the uptake of glucose into the liver, muscle and fat. Insulin is secreted from the endocrine pancreas in response to increased levels of circulating glucose, possibly through stimulating ENaC via phosphatidylinositol-3-kinase (PI3-kinase). Although a recent study on the mouse cortical collecting duct conducted by Mansley & Wilson (2010b) has shown that basal Na^+ transport can occur independently of the P13K pathway. This pathway seems to be essential for insulin induced Na^+ transport as it can be triggered by increased expression of α- and γ-ENaC in mouse renal tissue which is induced by insulin (Tiwari *et al.*, 2007). Insulin perfusion in the mouse renal tissue has been shown to increase the apical localisation of each ENaC subunit in cortical collecting duct principal cells (Song *et al.*, 2006). This is consistent with the finding of Wagner *et al.*, (2001), who suggested that insulin significantly induces PI3K and the protein to further increase Na^+ reabsorption through ENaC in A6 cells and cortical collecting duct cell lines via activation, likely phosphorylation, of SGK1 (Pearce, 2001; Wang *et al.*, 2001; Pearce & Kleyman, 2007). Insulin also appears to be involved in stimulating fluid clearance in diabetic patients (Guazzi *et al.*, 2002a; 2002b), which may be due to the control of pulmonary Na^+ transport (Hagiwara *et al.*, 1992). However, in the airway epithelial cells of H441, insulin-induced cells cannot exert such control over Na^+ transport, and the same is true for hormone-deprived cells; further augmentation of the natriferic effects by GC-induced cells is required (Brown *et al.*, 2008).

1.5.4.1 Possible mechanism of insulin

The activity of PI3K can be increased by several hormones / growth factors, including insulin, to activate the signalling cascade. This functions as a localised messenger and results in membrane localisation of the serine/threonine protein kinase B (PKB) and their pleckstrin homology (PH) domain (Alessi & Cohen, 1998).

PI3-kinase is characterised by its ability to phosphorylate plasma membrane phospholipids such as phosphatidylinositol-4,5 phosphate (PIP_2) and thus catalyse the formation of biologically active second messengers such as phosphatidylinositol-3,4,5 phosphate (PIP_3) (Rameh & Cantley, 1999). The increased abundance of PIP_3 in the plasma membrane results in the translocation of phosphoinositol dependent kinase (PDK1) to the membrane and this leads to increased phosphorylation of substances, which in turn catalyse the activation of Thr^{256} of SGK1 (Kobayashi & Cohen, 1999). The second messenger PIP_3 also activates TORC2 (Mora *et al.*, 2004), and this signalling complex, in turn catalyses the phosphorylation of SGK1 at Ser^{422} (Kobayashi & Cohen, 1999; Park *et al.*, 1999). This step is necessary as it allows PDK1 to phosphorylate the SGK1 protein at Thr^{256}, leading to full activation of SGK1 (Biondi *et al.*, 2001; Pearce *et al.*, 2010).

Insulin-induced Na^+ transport in A6 cells is the result of Na^+ channel density (Blazer-Yost *et al.*, 1998). Blazer-Yost *et al.* (1998) found that both PI3K and ENaC were co-localised as a result of insulin stimulation. This was thought to be dependent upon PI3K, as LY294002 prevented the natriferic response, as well as the translocation of the PI3K-ENaC complex to the membrane (Blazer-Yost *et al.*, 2003). They further speculated that the lack of the PI3K second messenger (PIP_3) was the cause of this. They were able to confirm this using confocal fluorescence microscopy which showed that the distribution of PIP_3 followed the translocation

of the PI3K-ENaC complex. Consequently, their model proposed that ENaC insertion into the membrane was a result of changes in lipid composition (Blazer-Yost & Nofzigar, 2004). In addition, $PIP_{2/3}$ can alter channel kinetics (Staruschenko *et al.*, 2007; Pochyunyuk *et al.*, 2007b) by augmenting ENaC activity by binding directly to ENaC subunits (Pochyunyuk *et al.*, 2007) which in results in Na^+ transport. However, these studies were conducted on renal epithelia and the effects were transient, indicating an acute regulation of ENaC. The PI3K inhibitor (LY294002) is now known to exert non-specific effects, therefore it has been advised that its use as an inhibitor be discontinued (Bain *et al.*, 2007). Nevertheless, these studies have extensively suggested a possible mechanism of action for insulin on ENaC via PI3K. This mechanism is SGK1 independent, therefore could explain why the *sgk1* gene knock-out mice do not display any overt lung phenotype. This leads to a possible role for insulin / PI3K in the control of Na^+ transport in the lung. Although this notion has received much less attention, Brown *et al.*, (2008) have conducted experiments on the effect of insulin on GC mediated Na^+ transport to further show that insulin could augment the effect of dexamethasone in the pulmonary epithelium. However, this is not the case in single H441 cells which produce different responses to groups of cells (Brown *et al.*, 2008). Insulin also has been linked to Na^+ transport in the foetal lung (Hagiwara *et al.*, 1992) and this hormone does appear to improve gas diffusion in diabetic patients (Guazzi *et al.*, 2002a; 2002b).

1.5.5 cAMP agonists

The other candidates for Na^+ regulation are cyclic Adenosine Monophosphate (cAMP) agonists. Initial studies conducted by O'Brodovich *et al.*, (1990) and Walters *et al.*, (1990) have examined the importance of cAMP in gestation-dependent absorption of foetal lung liquid. In kidney cells, vasopressin is known to elevate ENaC expression prior to the defence of hypovolemia, hypotension and increased plasma osmolality (Snyder, 2000; Gaeggeler *et al.*, 2011). In FRT epithelia expressing ENaC, cAMP agonists induce ENaC translocation towards

the cell surface and increase vesicle ENaC trafficking (Snyder, 2000). These agonists activate the Protein Kinase A (PKA) pathway which promotes Nedd4-2 interaction with the PY–motif of ENaC at the cell surface, via direct phosphorylation of Nedd4-2. This might elevate channel translocation, therefore in cases of Liddle's Syndrome such mutations at the PY-motif might disrupt cAMP-mediated stimulation. This stimulation has been shown to exist on both the mammalian kidney collecting duct and amphibian epithelia (Garty & Palmer, 1997), but it has not been confirmed in the distal colon (Garty & Palmer, 1997), the CFTR of human airway epithelia (Mall *et al.*, 1998) or in *Xenopus laevis* oocytes (Awayda *et al.*, 1996; Mall *et al.*, 1996). However, a series of studies have proposed that cAMP agonists can regulate Na^+ absorption in human lung cell lines such as H441 (Lazrak & Matalon, 2003; Clunes *et al.*, 2004; Inglis *et al.*, 2009). Hence, there is evidence to show that PKA, a downstream effector of cAMP, increases ENaC expression in the membrane (Snyder *et al.*, 2004a,b).

1.5.6 Nedd4-2

The ubiquitin ligase, neural precursor cell-expressed developmentally down-regulated protein 4 (Nedd4), is a ubiquitin ligase that can target ENaC channel complexes for internalisation / degradation by binding to specific sites within a C-terminal region of each ENaC subunit (Staub *et al.*, 1996; Staub *et al.*, 1997b; Dinudom *et al.*, 2001). Whilst Nedd4 was found to interact with ENaC *in vitro* (Staub *et al.*, 1996) and *in vivo* (Staub *et al.*, 1997b), the isoforms of Nedd4-2 revealed close conservation sites to Nedd4 (Snyder *et al.*, 2004b) but exhibited a higher affinity to bind to PY-motifs of ENaC subunits compared to Nedd4/1 (Kamynina *et al.*, 2001a; b).

When co-expressed with the ENaC subunits in *Xenopus laevis* oocytes Nedd4-2 has been shown to inhibit the ENaC-dependent Na^+ current by reducing the surface abundance of the channel subunits (Goulet *et al.*, 1998; Abriel *et al.*, 1999; Harvey *et al.*, 2001; Kamynina *et al.*,

2001a, b). The interaction between Nedd4-2 and ENaC is dependent on a conserved proline-rich motif, PPPXYXXL (or proline motif, PPXY) present in the C terminal region of each ENaC subunit that serves as tyrosine-based internalisation sequence (Chen & Sudol, 1995; Staub *et al.*, 1996; Staub *et al.*, 1997a,b; Farr *et al.*, 2000; Macias *et al.*, 2002; Ilsley *et al.*, 2002). The binding between Nedd4-2 and ENaC permits ubiquitination and this, in turn, serves as a marker for endocytosis and degradation (Staub *et al.*, 2000). Evidence that the interaction between ENaC and Nedd4-2 is physiologically important comes from naturally occurring mutations that disrupt the PY motifs in β- and γ-ENaC. These mutations cause Liddle's syndrome, a severe form of Na^+-sensitive hypertension that is due to excessive Na^+ retention via ENaC in the distal nephron, which is secondary to an increase in the apical abundance of ENaC, that reflects the impaired internalisation of these channels (Schild *et al.*, 1996; Dinudom *et al.*, 2001).

Further evidence for the role of Nedd4-2 *in vivo* was provided by a recent knock-out mouse model (*Nedd4-2* $^{-/-}$) that produced cystic-fibrosis (CF)-like symptoms in the airway and premature ENaC activation in the lung (Boase *et al.*, 2011). Nedd4-2 has also been shown to regulate ENaC in a ubiquitination-dependent manner in *Xenopus laevis* oocytes (Debonneville *et al.*, 2001; Konstas *et al.*, 2002; Snyder *et al.*, 2002; Fotia *et al.*, 2003; Ichimura *et al.*, 2005; Nagaki *et al.*, 2006; Hallows *et al.*, 2010; Zhang *et al.*, 2010) and epithelial cells (Snyder *et al.*, 2002; Zhou & Snyder, 2005; Ichimura *et al.*, 2005; Bruce *et al.*, 2008; Raikwar & Thomas, 2008; Zhang *et al.*, 2010, Kimura *et al.*, 2011) to indirectly increase ENaC expression at the cell surface, as a result of preventing the internalisation/ withdrawal of ENaC subunits from the membrane.

Nedd4-2 associates with ENaC by ubiquitinating all subunits (Zhou *et al.*, 2007; Kabra *et al.*, 2008; Eaton *et al.*, 2010). The association between Nedd4-2 and ENaC appears to be SGK1

dependent, as this stimulates ENaC activity, by phosphorylating Nedd4-2 to prevent binding to ENaC and therefore increasing the chance of ENaC being expressed at the cell surface (Chen *et al.*, 1999; Náray-Fejes-Tóth *et al.*, 1999; Alvarez de la Rosa *et al.*, 1999; Loffing *et al.*, 2001; Staub *et al.*, 2005). This can be demonstrated by mutating SGK1's PY-motif (Snyder *et al.*, 2002), which impairs its ability to bind to ENaC (Debonneville *et al.*, 2001; Snyder *et al.*, 2002). As a result, the ubiquitination process reduces ENaC at the plasma membrane and significantly decreases the Na^+ current, as found in *Xenopus laevis* oocytes and Fischer rat thyroid cells (Harvey *et al.*, 2001; Kamynina *et al.*, 2001a; McDonald *et al.*, 2002).

Prior to completing the interaction between Nedd4-2 and ENaC, the phosphorylation of Nedd4-2 is further stabilised by 14-3-3 protein binding (Ichimura *et al.*, 2005; Bhalla *et al.*, 2006; Liang *et al.*, 2006; Liang *et al.*, 2008; Liang *et al.*, 2010). The afore mentioned studies have reported that 14-3-3 is involved in this regulatory process and modulates the Na^+ current by cooperating with the SGK1 mediated response. They proposed that the inactive form of Nedd4-2 could increase the density of ENaC on the cell surface as 14-3-3 appears to be a binding partner of Nedd4-2 (Ichimura *et al.*, 2005). Therefore, the 14-3-3 family represents a novel class of Nedd4-2-binding proteins and affects the way that Nedd4-2 interacts with ENaC.

Cyclic AMP-coupled agonists can also promote the PKA-mediated phosphorylation of Nedd4-2 and this kinase appears to catalyse the phospho-sites in a similar way to the residues involved in the SGK1 pathway (Snyder *et al.*, 2004a). This has also been shown to be mediated by the binding of the 14-3-3 protein (Nagaki *et al.*, 2006) which triggers phosphorylation at specific residues of Ser/Thr (Muslin *et al.*, 1996). The data suggested that PKA and SGK1 congregate near Nedd4-2 so that they can phosphorylate it and therefore enhance ENaC at the cell surface. This notion is based on the phosphorylation of Nedd4-2 through the cAMP-activated PKA pathway in over-expressing SGK1 cells (Snyder *et al.*, 2004a). The concept of integration may

thus involve multiple pathways that are known to promote Na^+ transport and that are dependent on Nedd4-2 and 14-3-3 protein binding.

1.5.7 Cleavage by serine-proteases

A substantial body of work has shown that the activation of ENaC is also dependent on the cleavage of α- and γ-ENaC, by intracellular and extracellular proteases (see e.g., Vallet *et al.*, 1997; Vuagniaux *et al.*, 2000; Hughey *et al.*, 2003; Caldwell *et al.*, 2004; Hughey *et al.*, 2004; Caldwell *et al.*, 2005; Myerburg *et al.*, 2006; Diakov *et al.*, 2008; Passero *et al.*, 2008; Rossier & Stutts, 2009; Myerburg *et al.*, 2010; Tan *et al.*, 2011). The cleavage occurs during channel maturation within the biosynthetic pathway, which involves the cleavage of both the α- and γ-subunits by furin at the serine residue (Kellenberger *et al.*, 1999a;b; Vallet *et al.*, 1997; Kashlan *et al.*, 2005). However, cleavage can also occur in the extracellular space, prior to full activation of the ENaC subunits. This was first suggested by Hughey and colleagues, when they detected a mixture of mature and immature ENaC in the pool of plasma from the surface of stably transfected Madin-Darby canine kidney cells (MDCK) (Hughey *et al.*, 2004a, b). They demonstrated that the immature channel complexes at the plasma membrane with a reserve of poorly functional channels needed to be activated by proteases in post-Golgi compartments. They also observed that both α- and γ- subunits have consensus sites for cleavage by furin, a serine-protease family protein, or other serine proteases such as prostasin and trypsin, at the cell surface prior to ENaC activation (Hughey *et al.*, 2004a; b). However, it is possible that either individual channels at the surface undergo maturation through the cleavage process, or that some subunits escaped maturation, producing a mixture of both mature and immature subunits (Hughey *et al.*, 2004a; b). These events contribute to ENaC residency in the membrane (see review, Butterworth *et al.*, 2009, 2010).

Whilst it has been suggested that both α- and γ-ENaC are cleaved prior to activation, this is not the case for the β-subunit (*Figure 1-8*). The β-subunit is not thought to be subject to cleavage because it is insensitive to both endogenous and exogenous proteolytic cleavage. One may, however, speculate that the *N*-glycosylation of the β-subunit has fewer than 12 *N* glycosylation sites (Harris *et al.*, 2007) which could protect the proteolytic cleavage sites from enzyme access to their substrate. In addition, the primary sequence of the β-subunit does not have any consensus sequences for furin cleavage. The discrepancy in primary sequence and protein folding / maturation might explain the striking difference between the β- subunit and the α- and γ-subunits (Harris *et al.*, 2007).

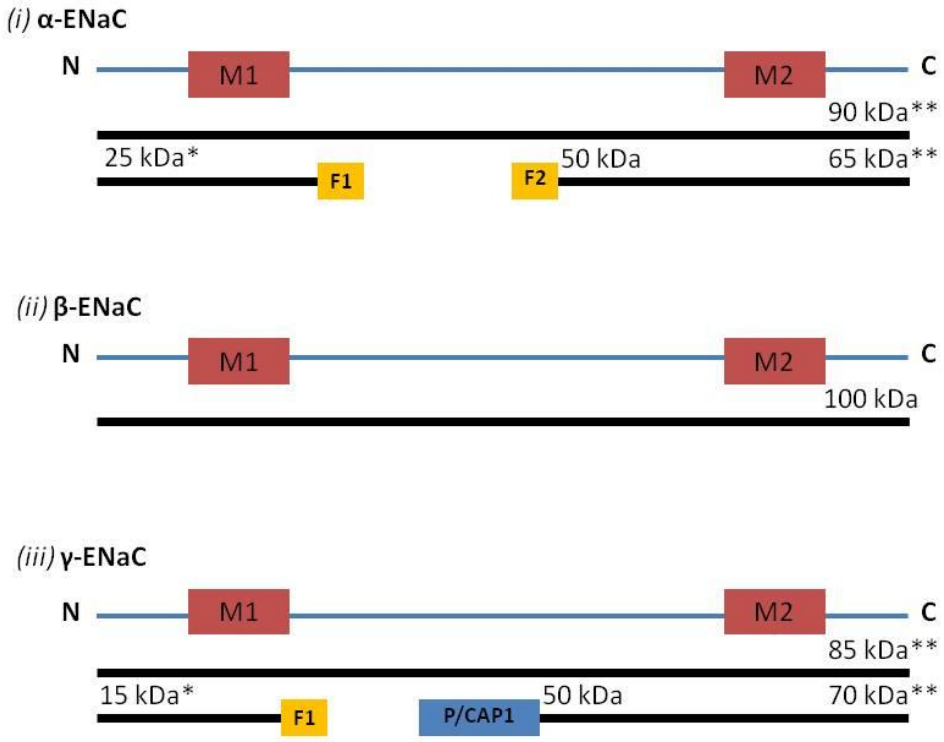

Figure 1-8: Prediction of linear models for ENaC subunit cleavage (adapted from Rossier & Stutts, 2009)

Each subunit has common features, including two transmembrane domains **(M1 and M2)** with short **N- and C- termini**, known and suspected sites of ENaC cleavage, and that have been detected by N-terminal (*) or C-terminal (**) antibodies. (*i*) **α-ENaC** contains two possible furin sites, **F1 and F2** which generate **25, 50, 65 and/or 95 kDa** fragments as detected by an N-terminal antibody; (*ii*) **β-ENaC** does not contain any furin sites and other cleavage sites are postulated to be protected by heavy glycosylation, therefore the antibody raised to the N- and C- terminal will generate approximately **100 kDa**; (*iii*) **γ-ENaC** contains **furin, prostasin (P) and channel activating proteases (CAP)** sites, therefore it will generate approximately **15, 50, 70, and/or 85kDa** fragments.

The models suggested by the Hughey, Kleyman and Harris groups are complementary to studies of heterologously expressed ENaC subunits incorporating synthetic epitopes (*eg:* FLAG) that facilitate the identification of cleavage products. Proteases / protease inhibitors clearly modify electrogenic Na^+ transport in absorptive cells / tissues (see e.g. Myerburg *et al.*, 2006; Lazrak *et al.*, 2009; Tan *et al.*, 2011). In many epithelia, ENaC appears to be constitutively activated through the endogenous serine proteases because basal transepithelial Na^+ transport cannot be further activated by the addition of trypsin. However, the addition of aprotinin (protease inhibitor) on the apical side of the epithelium typically inhibits baseline Na^+ transport; this effect is reversible by exposing the cells to trypsin. In *Xenopus* kidney cells, aprotinin can block up to 90% of amiloride sensitive electrogenic Na^+ transport (Vallet *et al.*, 1997; Adebamiro *et al.*, 2005) and 50% in the mouse cortical collecting duct cell line (mpk-CCD_{c14}) (Vuagniaux *et al.*, 2000), whereas aprotinin inhibited 30% of the basal I_{Na+} current in human nasal cells (Bridges *et al.*, 2001). Channel activating proteases (CAPs) have been shown to be expressed in tissues including the distal lung epithelium (Vallet *et al.*, 2002) and found in the secreted lung liquid (Verghese *et al.*, 2004; Planes *et al.*, 2005). A lack of the *CAP1* gene in mice has been shown to inhibit lung fluid clearance by β-adrenergic agonists; however, the effect was reversed by treatment with a non epithelial soluble serine protease (Planes *et al.*, 2009). This could be taken as further evidence of the synergistic action of hormones in the control of fluid clearance, where GCs and thyroid hormones prime the lung for fluid clearance and epinephrine/adrenaline causes activation of ENaC in the membrane via a mechanism of proteolytic cleavage to increase channel activity.

1.6 Closing remarks

This chapter has provided a basic insight into the regulation of lung fluid homeostasis. The importance of ENaC can be seen through the knockout studies of α-ENaC in mice that resulted in death due to a failure to clear the lungs of fluid. Most studies have pointed out that α-ENaC activation for lung fluid clearance must be mediated by SGK1. However, this remains controversial because mouse models that lack this gene (*sgk1*$^{-/-}$) are viable and show near normal fluid homeostasis in the lung. Thus, the role of SGK1 is not fully understood and requires further examination. Investigation into the role of SGK1 can be performed using inhibitors that have been specifically developed to block the protein kinase activities that underlie the SGK1 pathway, including the PI3K and TORC2 regulation of SGK1 activity. When SGK1 is active, it must phosphorylate an ubiquitin ligase protein, Nedd4-2, which is crucial for maintaining ENaC at the cell surface.

Such activation of ENaC can also be regulated by the cAMP activating PKA pathway. Whilst this is activated by different hormones, this pathway must converge at Nedd4-2, which serves as a general mechanism to ubiquitinate ENaC prior to internalisation. If this is true, Nedd4-2 may serve as a useful tool to observe how both SGK1 and PKA affect ENaC surface abundance. Furthermore, we also need to distinguish whether PKA acts alone in ENaC regulation or via SGK1.

1.7 Thesis Aims

The main aim of the proposed study is to determine how hormones alter ENaC subunit recruitment to the cell surface of the H441 cell line. The relative expression of each subunit of ENaC will also be observed subject to hormonal stimulation by dexamethasone and cAMP elevating drugs. Further questions that may arise are summarised in *Figure 1-9* and as follows:

1) Does dexamethasone increase the surface expression of ENaC subunits by activating the PI3K - SGK1 pathway?
2) Is the ubiquitin ligase Nedd4-2 dependent on SGK1 to maintain ENaC at the cell surface?
3) Do cAMP activating drugs affect ENaC subunits through the PKA pathway?
4) If so, do cAMP activating drugs involve Nedd4-2?
5) Is Nedd4-2 subject to the mechanisms of both the SGK1 and PKA pathways?

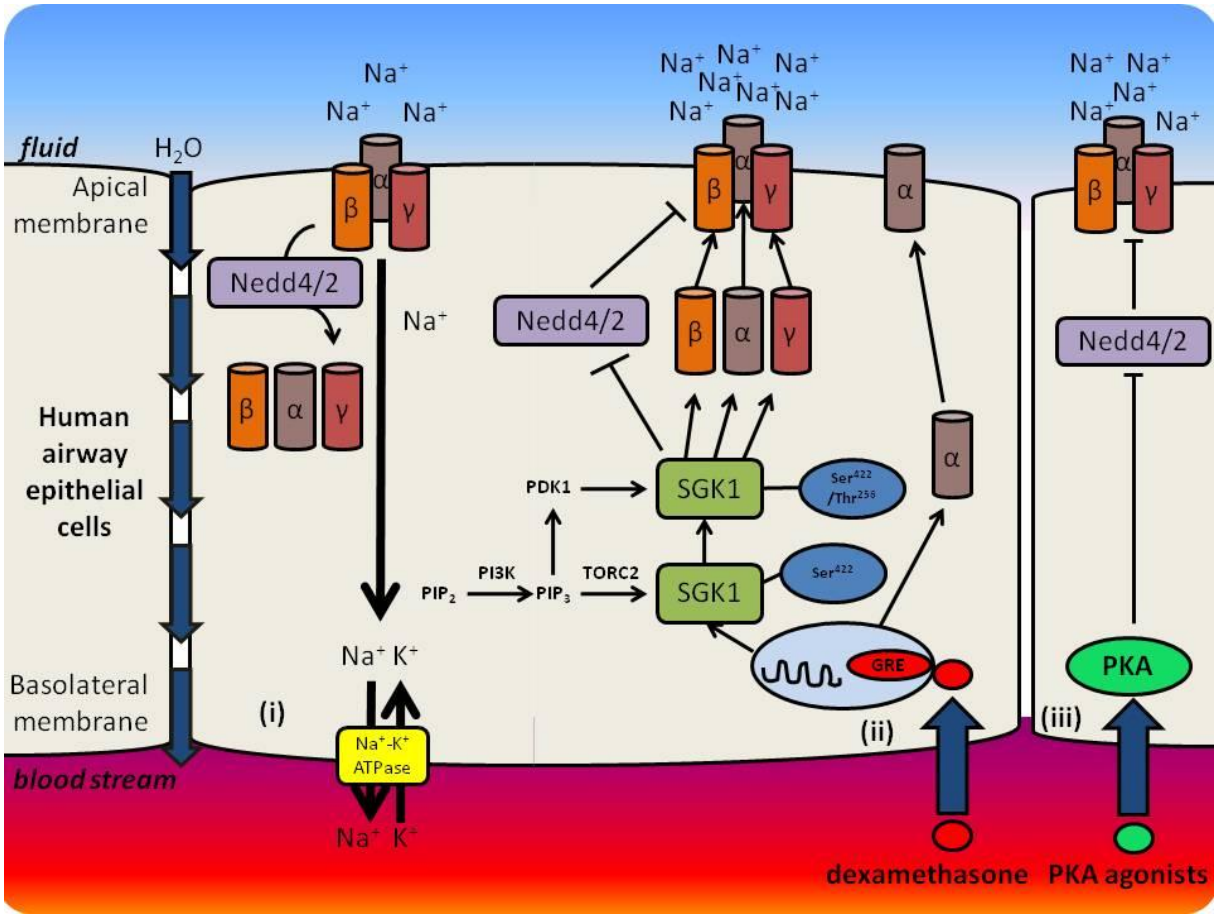

Figure 1-9: Diagram showing the regulation of ENaC in three different conditions

(i) basal condition, where ENaC subunits at the cell surface are normally expressed and further internalised by Nedd4-2; *(ii)* activation of ENaC and deactivation of Nedd4-2 by the effect of dexamethasone, through SGK1 activity and *(iii)* activation of ENaC and deactivation of Nedd4-2 by the cAMP elevating PKA pathway.

1.8 Thesis overview

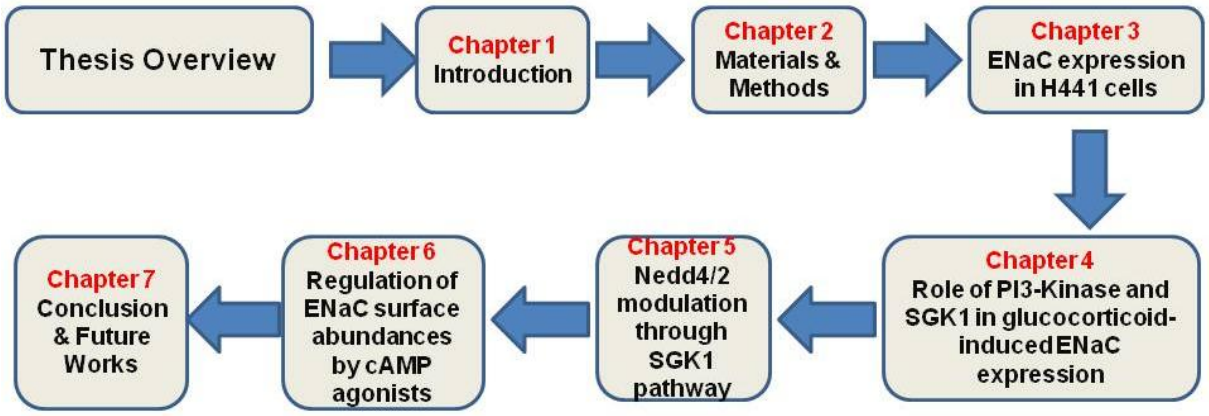

Figure 1-10: General thesis overview

This thesis presents a study on the expression of epithelium sodium channels, ENaC. Here I examine in detail the expression of each subunit and explore the potential pathways involved in the modulation of the endogenous expression of ENaC in the human epithelial airway cell line, H441.

A literature review is presented in *Chapter 1*. The first part of the chapter offers a general description of Na^+ transport in epithelial airway cells through ENaC. This description includes information on the functional structure of ENaC at the lung epithelial cell surface subject to hormonal responses. Next, the chapter focuses on ENaC activation through the glucocorticoid-induced pathway which specifically involves PI3K-TORC2-SGK1-Nedd4-2, and enhances ENaC expression by protein trafficking from the intracellular pool of proteins towards the cell surface. This activation is also thought to be mediated by protein kinase A (PKA pathway). Since the information provided in this chapter is more of a general introduction, it will be supplemented by a specific introduction at the beginning of each small project, thereby linking together the four main chapters (3, 4, 5, 6).

Before proceeding to the main chapters of the thesis, the general methodology is described in *Chapter 2*. This describes the experimental procedures that were adopted throughout the thesis, including each solution/chemical/antibody where relevant. The introduction to each chapter is then followed by a short section detailing the experimental design and clarification of the aims and objectives for each main chapter.

The first project begins in *Chapter 3*. I initially aim to investigate the characteristics of the endogenous ENaC subunit proteins. I explore the expression of each subunit from the intracellular and cell surface pool of proteins in presence of dexamethasone.

Preliminary data is then collated for significance. In *Chapter 4* I investigate the modulation of ENaC through protein kinase signalling up-stream and down-stream of the SGK1 pathway to confirm a role for dexamethasone in the physical stimulation of Na^+ transport in H441 cells (Clunes *et al.*, 2004; Ramminger *et al.*, 2004; Husband, 2006; Brown *et al.*, 2008; Gallacher *et al.*, 2009; Watt *et al.*, 2012). The relative expression of ENaC between glucocorticoid-deprived and glucocorticoid-treated cells, provides the answer to an intriguing question: Does dexamethasone participate in glucocorticoid regulated kinase (SGK1) activation to assist ENaC translocation from the intracellular compartment to the cell surface? The pharmacological compounds PI-103, TORIN1, GSK650394 and rapamycin are used to explore the role of SGK1 in ENaC expression.

To further confirm these results, *Chapter 5* describes the expression of a ubiquitin ligase protein, Nedd4-2, under the influence of dexamethasone. This hormone is thought to stimulate SGK1 expression prior to the phosphorylation of Nedd4-2 (Debonneville *et al.*, 2001; Snyder *et*

al., 2002). Therefore, it would be interesting to determine the correlation between Nedd4-2 and ENaC protein subunits expression on cell surface through SGK1 pathway.

Chapter 6 investigates the role of cAMP agonists on the elevation of ENaC expression by activating Nedd4-2, which is also thought to be dependent on the PKA pathway. Thus the findings complement the physiological ENaC activity under these treatments (Clunes *et al.*, 2004; Woollhead & Baines, 2006) which may be a subject to the convergence point of Nedd4-2 (Snyder *et al.*, 2004a).

Finally, each of the main chapters concludes with a discussion of the findings from that chapter. *Chapter 7* concludes the thesis, containing a summary of the completed work, a general discussion of the findings and suggestions for future work.

2

Materials & Methods

2.1 Methods overview

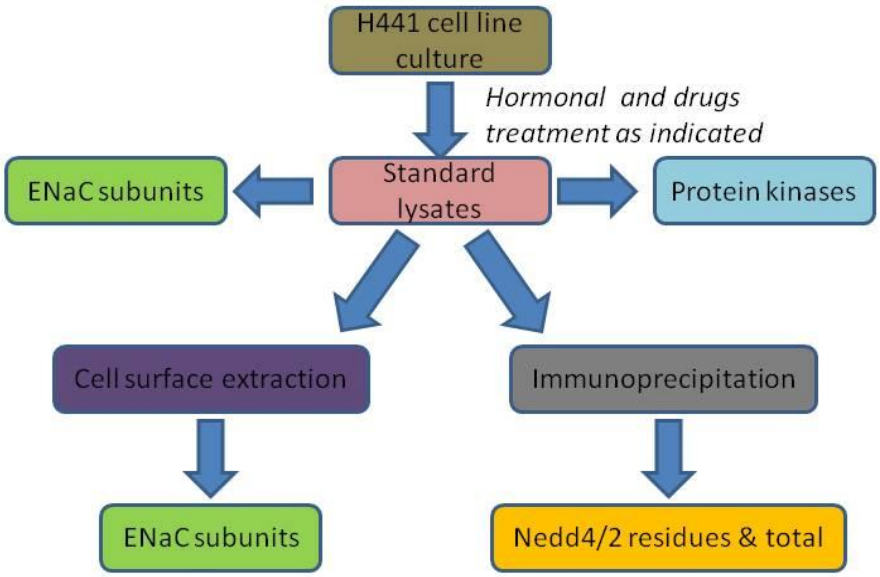

Figure 2-1: A methodology overview, as applied throughout the thesis

This chapter describes the methods applied throughout the thesis, starting with the extraction of the H441 cell line, the detection of ENaC subunits and the expression of the Nedd4-2 protein (*Figure 2-1*). Once the H441 cells reached confluence, they were treated with hormones and drugs as appropriate. Then, the cells were extracted through a standard protein extraction, subjected to Western analysis to detect ENaC subunit expression and the protein kinases involved in the P13K, SGK1 and PKA pathways. Subsequently, another two crucial protocols

were applied to the cell lysates: *(i)* cell surface extraction through biotinylation and *(ii)* immunoprecipitation of the Nedd4-2 protein. Each of the protocols were processed using Western analysis and probed for ENaC subunits and Nedd4-2 protein, respectively.

2.2 H441 cell line culture

2.2.1 The H441 cell model

The H441 human bronchiolar epithelial cell line was used throughout the experiments. The cell line was purchased from The American Type Culture Collection (ATCC) and had been originally isolated through pericardial effusion from a male patient suffering from a pulmonary adenocarcinoma in May, 1982 (Gazdar *et al.*, 1990). The cells form an adherent monolayer therefore they are suitable for cell culture. A characteristic of the H441 cell line is that it contains cytoplasmic granules that resemble those found in bronchiolar Clara cells (Gazdar *et al.*, 1990) which constitute about 80% of the cell population of the distal airways (Kulaksiz *et al.*, 2002). The authors have also shown that the cell line has the ability to express RNA for surfactant protein A and B. They also reported that these cells can be activated by cAMP agonists to generate a Cl^- conductance (Kulaksiz *et al.*, 2002). Despite this, the cells display an absorptive phenotype with additional treatment of the synthetic glucocorticoid, dexamethasone (Lazrak & Matalon, 2003; Clunes *et al.*, 2004; Ramminger *et al.*, 2004; Thomas *et al.*, 2004; Shlyonsky *et al.*, 2005; Brown *et al.*, 2008; Inglis *et al.*, 2009; Watt *et al.*, 2012), suggesting that they are a useful model for investigating the activation of ENaC through hormonal control in the human distal airway.

2.2.2 Media

RPMI-1640 was developed by Moore *et al.*, (1966) at the Roswell Park Memorial Institute, hence the acronym RPMI. The formula is based on the bicarbonate buffering system and

alteration of the amounts of amino acids and vitamins. The medium has been used for the culture of human normal and neoplastic leukocytes. When properly supplemented, it has been shown to support the growth of many types of cultured cells, including fresh human lymphocytes in the 72 h phytohemaglutinin (PHA) stimulation assay.

The H441 cells were first brought up in RPMI 1640 medium (Sigma Aldrich, Poole, UK) with foetal bovine serum (FBS) and bovine calf serum (BCS) as main supplements. For the experiment, the growth medium then was changed to a fully defined medium identical to that described in *Table 2-1,* except that the insulin concentration was reduced to 20 nM, and the serum components were replaced with FBS (8.5%) that had been dialysed to remove hormones/growth factors. Each supplement was stored in aliquots to minimise thaw-refreeze and contamination in the media. All components were stored in the freezer (-20^{o}C) and were thawed in a 37^{o}C water bath prior to use.

The cells were maintained in a humidified incubator at 37^{o}C and exposed to 5% CO_2, 13% O_2, and 82% N_2 to stimulate the conditions found in the distal airways, where the P_{O2} is approximately 100 mmHg (West, 2000). To ensure a tightly defined hormone condition during the experiments, cells were bathed in a defined media for at least 24 hours before conducting the experiments. For some experiments, dexamethasone (200 nM) was added to the media for 24 h and others for 3 h in order to explore the effects of both long and brief stimulation with the hormone. Throughout the experiment, appropriate age-matched control cells lacking the hormone were also studied.

Table 2-1: Composition of components in both complete and dialysed media

Supplement	Function	Complete Media	Dialysed Media
Bovine Calf Serum (BCS)	Contained a mixture of Bovine Serum Albumin (BSA) and growth hormones to allow eukaryotic cells to survive, grow and divide	8.5%	-
Foetal Bovine Serum (FBS)	Same as above	8.5%	-
Dialysed FBS	As above except growth hormones were dialysed to minimise the effect of hormones in the experiment.	-	8.5%
Glutamine	Supports energy to the cells whenever the energy demands are high.	2 mM	2 mM
Transferrin	An inert energy to facilitate iron in the cells (Fletcher & Huehns 1968)	5 µg/ml	5 µg/ml
Selenium	Essential trace element to prevent oxidative damage in the cells.	5 ng/ml	5 ng/ml
Antibiotic/Antimycotic	Reduces the growth of bacteria and fungus by inhibiting bacterial/fungus protein synthesis (Pearlman 1979)	0.2%	0.2%

All supplements were purchased from Sigma Aldrich, Poole, UK except BCS (from Gibco, Paisley, UK).

2.2.3 Routine culture

Cells were routinely cultured in a 75 cm^2 filter capped culture flask (Greiner Bio-one, Frickenhausen, Germany) using complete media per passage per week. Each passage was approximately 95% confluent after one week. Cells were dissociated from the flask using warm trypsin/EDTA solution (Sigma Aldrich, Poole, UK); then fresh complete media was added to the flask to deactivate the dissociation. Cells were suspended using low spin centrifugation (500 x *g* for 3 min) and the density was determined using disposable plastic haemocytometers (ISL "Fast Read", Paignton, UK). 10^6 cells were plated into a fresh flask containing fresh complete media. The volume of cells varied according to the receptacle used: in flasks, 6-well plates (0.5×10^6), transwells (1.0×10^6) and petri dishes (1.0×10^6). The media was changed every other day unless mentioned otherwise.

2.3 Preparation of samples

2.3.1 Standard lysates

H441 cells were routinely cultured in a complete medium until confluent. 24 h before an experiment, a fully defined medium supplemented with dialysed FBS was added after the complete medium was removed. Then, the confluent cells (~80-90%) were exposed to pharmacological agents (*Table 2-2*) in fully defined media. After the required incubation time, cells were washed three times with ice-cold phosphate buffered saline (PBS) to remove any agent traces. Cells were then resuspended in lysis buffer (*Table 2-3*). Each sample was then sonicated for 10 seconds ("Soniprep 150" MSE Ltd, Kent, UK) and further spun at 14,000 x *g* for 10 min. The concentration of crude protein was determined by the Bradford method. 2 x SDS sample buffer (*Table 2-4*) was added to make 1 x of the final concentration. The samples were heated at 95^{o}C for 5 min to denature the protein. The cells were then loaded onto the gel at the respective protein mass per well or kept in the -80^{o}C freezer for further use.

Table 2-2: Pharmacological agents involved throughout experiment

Compound	Function	Vehicle	Final concentration	References
Insulin*	Growth hormone	ddH_2O	20 nM	Clunes *et al.*, 2004; Mansley & Wilson, 2010 a,b
Dexamethasone*	Glucocorticoid hormone	Basal media	0.2 µM	Brown *et al.*, 2008; Mansley & Wilson, 2010 b
TORIN1***	TORC 1/2 inhibitor	DMSO	0.1 µM	Guertin & Sabatini 2007; Thoreen *et al.*, 2009
PI-103**	PI-3 kinase pathway inhibitor	DMSO	1 µM	Bain *et al.*, 2007; Folkes *et al.*, 2008
GSK650394**	SGK1 pathway inhibitor	DMSO	10 µM	Sherk *et al.*, 2008
Rapamycin**	TORC1 inhibitor	DMSO	0.1 µM	Bain *et al.*, 2007; Thoreen & Sabatini, 2009
Forskolin*	cAMP agonists	DMSO	10 µM	Ramminger *et al.*, 2004; Inglis *et al.*, 2009
Isobutylmethylxanthine (IBMX)*	cAMP agonists	DMSO	100 µM	Clunes *et al.*, 2004
N^6, 2'-*O*-dibutyryladenosine 3'5'-cyclic monophosphate (bucladesine)*	cAMP agonists	ddH_2O	1 mM	Clunes *et al.*, 2004

Pharmacological agents purchased from:

*Sigma Aldrich, Poole, UK, ** Merck, Beeston, UK, ***TORIN1 was a kindly gift from Professor D. Sabatini, Whitehead Institute for Biomedical Research, US

Table 2-3: Composition of cell lysis buffer

Component	Concentration	Function
Tris-HCl pH 7.5	50 mM	pH buffer
EGTA	1 mM	Ca^{2+} chelating agent
EDTA	1 mM	Ca^{2+} chelating agent
Sodium Orthovanadate	1 mM	Broad spectrum inhibitor of protein tyrosine phosphatases
Glycerol Phosphate	10 mM	Competitive inhibitor of Ser/Thr and Tyr phosphatases
Sodium Pyrophosphate	5 mM	Competitive inhibitor of pyrophosphatases
Sodium Fluoride	50 mM	Ser/Thr phosphatase inhibitor aids protein stability
Sucrose	270 mM	Stabilise lysosomal membranes to reduce protease release
Triton x 100	1% (v/v)	Non-ionic detergent to solubilise proteins
β-Mercaptoethanol	0.1% (v/v)	Reducing agent, cleaves disulphide bonds
"Complete mini" protease inhibitor cocktail tablets*	1 tab per 10 ml	Protease inhibitors to block wide spectrum of protease activity

The components are all purchased from Sigma Aldrich (Poole, UK) except *Roche Molecular Biochemicals (Lewis, UK)

Table 2-4: SDS gel loading buffer (2 x concentration)

Components	Concentration
Tris.HCl (pH 6.8)	50 mM
SDS	2% (v/v)
Bromophenol blue	0.1% (v/v)
Glycerol	10% (v/v)
Dithiothreitol	100 mM

2.3.2 Cell surface extraction

Cells were seeded in a 10 cm^2 petri dish in *complete media*. The media was then added the following day and changed every alternate day until the cells had grown to confluence. The dexamethasone (0.2 µM) was then added for a duration of either 3 h or 24 h. Further incubation with pharmacological compounds was performed in the 3 h prior to extraction, unless otherwise stated (*Table 2-2*).

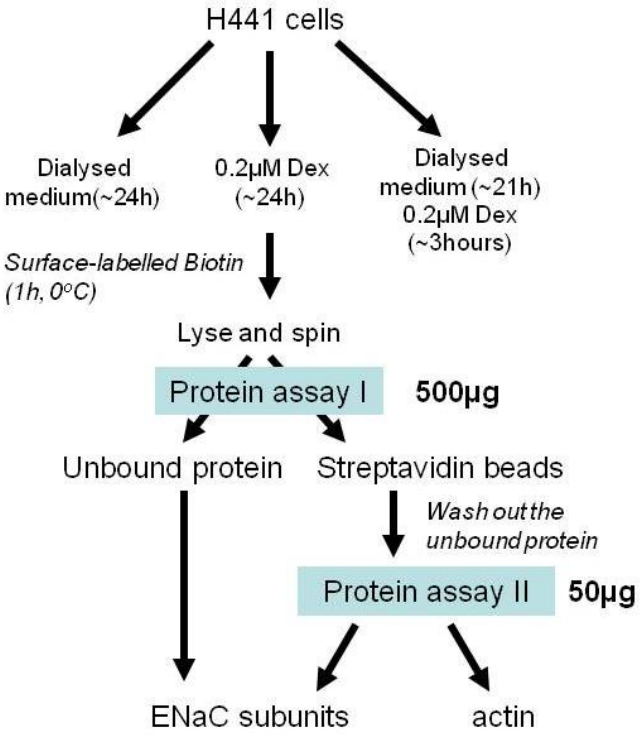

Figure 2-2: Cell surface extraction protocol

This protocol was initially carried out and modified from Planés *et al.*, (2002) and Harris *et al.*, (2007) (*Figure 2-2*). This was conducted on ice (0^oC) to fix and reduce the chances of surface protein being internalised. The cells were washed with ice-cold PBS twice to remove any media residue. Then the cells were exposed to 10 mM sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3' dithioproprionate (EZ-Link Sulfo-NHS-SS-Biotin, Pierce, Fischer Scientific, UK) to label the

surface for ~1 h then gently agitated. The reagent covalently modifies the proteins by attaching a cleavable biotin moiety to an amine group on the N-terminal amino acid residue and lysine side chains. The biotinylation reaction was quenched by adding cold glycine (100 mM). The cells were scraped in glycine then they were spun down, and the cells were lysed as described above. Then the lysates were further spun at 14,000 x *g* for 30 mins at 4^{o}C to precipitate the cell debris / insoluble proteins. The protein contents were then determined using Bradford reagent (Protein assay I) (Bio-Rad, Hemel Hempstead, UK). Protein that was extracted in this way is referred to as "total lysates" in the thesis.

In the initial series of experiments, 500 µg of total lysates were mixed with streptavidin-coated agarose beads (Pierce, Fischer Scientific, UK) and allowed to equilibrate for 60 mins at room temperature with continual agitation. Streptavidin beads bind the biotins with a high affinity which implies that the proteins that have been modified by the sulpho-NHS-SS-biotin reagent. The beads were then washed extensively to remove the unbound proteins and heated to 95% under strongly reducing / denaturing conditions (5% β-mercaptoethanol / 2% SDS) to cleave the disulphide bonds in the biotin moiety and release the bound proteins. The protein released in this way is referred to as "biotinylated protein". These experiments were repeated (n = 8) and measurement was performed using the protein assay RC-DC (RC-DC reagent, Bio-Rad, Hemel Hempstead, UK) (Protein assay II). The results showed that 48 ± 0.69 µg of protein could be recovered from an initial 500 µg. Since the binding capacity of the streptavidin-coated beads is 1-3 mg per ml per slurry, all subsequent experiments used 150 µl of slurry to isolate the biotinylated protein from 500 µg. Under these conditions there is a ~ 3 fold excess binding capacity and the smaller bead volume allows the biotinylated proteins to be released directly into 50µl of the sample buffer used for SDS-polyacrylamide gel electrophoresis.

Western analysis showed that β-actin was readily detectible in 1 μg aliquots of total protein and, although this protein could also be detected in large aliquots (20 μg) of biotinylated protein (see *Chapter 3*), it was necessary to overexpose the blots in order to detect this protein. Since β-actin is a cytoskeletal protein that is confined to the inner leaflet of the plasma membrane, this protein would normally be inaccessible to biotinylation reagents in the extracellular fluid. The presence of β-actin in the biotinylated protein fraction thus shows that this protein pool does contain some intracellular proteins. However, densitometric analysis showed that the abundance of β-actin in a 20 μg aliquot of biotinylated protein was ~30% of the abundance measured in 1 μg of total protein. The biotinylation-streptavidin binding protocol therefore allows surface-exposed proteins to be isolated with 98 – 99% purity, and this protein fraction is subsequently referred to as the "surface-exposed protein". The protein assayed was loaded into an SDS-Page 8% gel for ENaC subunit protein expression whilst 10% was used for endogenous phosphorylation of protein kinases. In all subsequent experiments aliquots of total protein (50 μg) and of the surface-exposed protein purified from 500 μg of total protein were subjected to Western analysis using antibodies against α-, β-and γ-ENaC. Cell surface labelling was restricted to plasma membrane proteins, as shown by the absence of any significant amount of actin in this fraction. Essentially, the non-biotinylated protein pool is characterised by a large amount of actin.

2.3.3 Immunoprecipitation of total Nedd4-2

This protocol was used to extract protein bound specifically to anti-Nedd4-2. The idea is to isolate the protein of interest through specific binding of the antibody (in this case, anti-Nedd4-2) to the antigen (Protein G). This technique is used because the total amount of cellular Nedd4-2 protein may be very small, therefore prohibiting detection by standard Western techniques. Rapid degradation of Nedd4-2 may also prevent detection; however this was

avoided thanks to the suggestions made by Professor Carol MacKintosh (University of Dundee).

As shown in *Figure 2-3*, a standard protein extraction of 1 mg of the protein lysates obtained using the Bradford protein assay was added to 20 µl of pre-washed Protein G Sepharose beads (Sigma Aldrich, Poole, UK) and incubated for 1 h at 4°C. This is to allow clear lysate binding to the Sepharose G beads. The cleared lysates were then exposed to Sepharose G beads that had been coated with 10 µg of anti-Nedd4-2 for 1 hr at 4°C while being continually agitated. All volumes were made up to 1 ml. Then, the bead-bound complexes were harvested by centrifugation at 14,000 x *g* for 5 min. The beads were then sequentially washed, first with a high salt buffer to reduce ionic interaction, then a no-salt buffer to reduce non-specific reactions (*Table 2-5*).

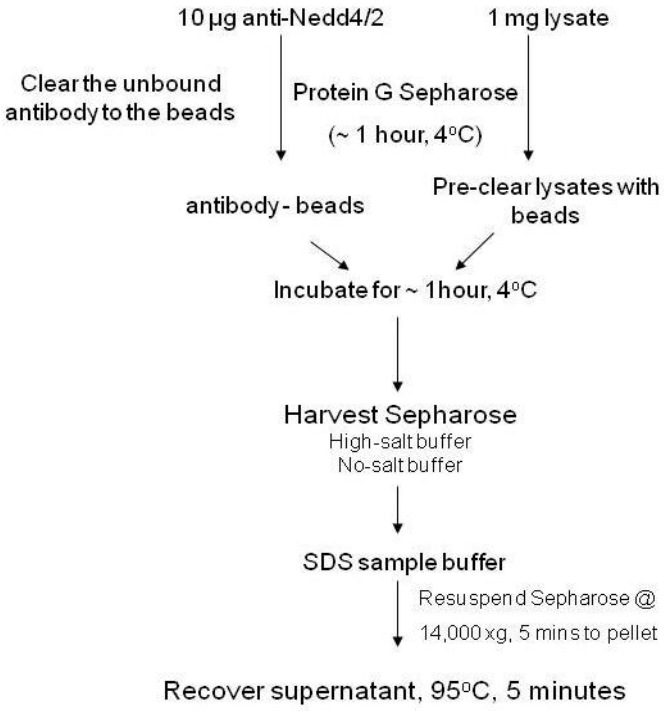

Figure 2-3: The immunoprecipitation protocol

The complexes were then centrifuged at 1000 x *g* for 1 minute, and this was then repeated twice. Following that, the beads were eluted by adding 20 µl of 2 x SDS sample buffer and heated up to 95^{o}C for 5 minutes. Finally, the protein-bead mixture was collected by spinning at 14,000 x *g* for 5 min and deionised water was added to make a final concentration of 1 x SDS sample buffer. They were subsequently fractioned using a 6% SDS polyacrylamide gel and Western Analysis, then transferred to Hybond-P membranes (Amersham, Bucks., UK) that were probed using antibodies against the Ser^{221}-, Ser^{327}-, or the Thr^{246}-phosphorylated forms of Nedd4-2. Blots were then stripped and re-probed using antibodies against the full length protein.

Table 2-5: Recipe for the wash buffer in the immunoprecipitation

High-salt wash buffer	No-salt wash buffer
2.5 ml 2M Tris.HCl pH7.5 12.5 ml 4M NaCl 85 ml deionised water	2.5 ml 2M Tris.HCl pH7.5 200 µl 0.5M EDTA 100 µl β-mercaptoethanol 97.2 ml deionised water

2.4 Western analysis

2.4.1 Protein determination by the protein assay

2.4.1.1 Principle of the assay

A protein assay was used to determine the concentration of soluble protein in the sample. From this, the protein mass can be calculated to obtain the amount desired for further analyses. The experiment assesses the concentration of protein using a dye, and measures the colour change at

an absorbance of 595 nm. This measurement indicates the extent of protein binding and thus the protein concentration.

2.4.2 Performing the assay

2.4.2.1 Bradford assay

Bovine serum albumin (BSA, Sigma Aldrich, Poole, UK) was prepared in a variety of concentrations: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 mg/ml in deionised water (ddH_2O). For each assay reaction, 5 µl of each BSA standard concentration were added in triplicate to a 96-well microtiter plate. 5 µl of deionised water were also added in triplicate to act as a control. 1 µl of each sample was then added in duplicate.

Protein Assay Dye Reagent Concentrate (Bio-Rad, Hemel Hempstead, UK) was diluted to a ratio of 1:4 with deionised water and filtered through Whatman no. 1 filter paper. 100 µl of the diluted dye reagent was added to each protein standard, control and sample for 10 minutes. The reaction was measured at an absorbance of 595 nm using a microplate reader (MRX, Guernsey, UK). Absorbances of all the known protein standards obtained were recorded in an Excel worksheet in order to produce a standard curve. The absorbance values for the protein lysates of the unknown samples were calculated using the standard equation ($y = mx + c$), where m is the gradient of the line and c is where the line intercepts the y-axis.

2.4.2.2 Modified Lowry assay (RC-DC assay)

A series of concentrations of bovine serum albumin (BSA, Sigma Aldrich, Poole, UK) were prepared as follows: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 mg/ml in SDS-PAGE buffer, as each of corresponding unknown protein sample was processed in similar buffer. This buffer contains SDS and is reducing agent compatible (RC) and detergent compatible (DC); RC-DC reagent. 25 µl of each protein standard, the control (SDS-PAGE buffer) and treated samples were

divided into separate 1.5 ml microcentrifuge tubes. 125 µl of RC-Reagent I was added to each tube. Each solution was mixed thoroughly. After that, 125 µl of RC-Reagent II was added and mixed. The reaction was centrifuged at 14,000 x *g* for 5 min. The supernatant was then discarded. 5 µl of DC-Reagent S was added to 250 µl of DC-Reagent A. 127 µl of this solution was added to each tube that contained a pellet. The reaction was incubated at room temperature for 5 min, or until the precipitation dissolved. The tubes were then vortexed before adding 1 ml of DC Reagent B and incubated for another 15 min. The absorbance (750 nm) was measured using a microplate reader (Dynatech Laboratories, UK). All values obtained were calculated using Excel. A standard curve was then produced and the protein mass for each of the samples was determined.

2.4.3 Gel casting

SDS – polyacrylamide gels were prepared according to the recipe modified from Harlow & Lane (1988). In this thesis, three different percentages of resolving gels were used for better protein separation (*Table 2-6*). A solution of resolving gel was poured into glass plates (Bio-Rad, Hertforshire, UK) with 1 mm spacers and allowed to set with 500 µl of butanol added to the surface to ensure removal of air bubbles. Once the gel had set, the butanol was washed out thoroughly and the stacking gel was poured in (*Table 2-7*). A comb was inserted to the stacking gel and left to set so that it could be used for electrophoresis straight away or kept in a refrigerator in damp tissue paper.

Table 2-6: Recipe for resolving gels

Solution components of resolving gels	Component volumes (ml) per 2 gels		
	6% of SDS	8% of SDS	10% of SDS
Deionised water	7.9	6.9	5.9
30% acrylamide mix	3.0	4.0	5.0
1.5M Tris (pH 8.8)	3.8	3.8	3.8
10% SDS	0.15	0.15	0.15
10% ammonium persulfate	0.15	0.15	0.15
TEMED (*N*,*N*,*N*',*N*'-Tetramethylethylenediamine)	0.012	0.50	0.5

Table 2-7: Recipe for 5% SDS of stacking gels

Components	Component volumes (ml) per 2 gels
Deionised water	2.1
30% acrylamide mix	0.5
1.5M Tris (pH 8.8)	0.38
10% SDS	0.03
10% ammonium persulfate	0.03
TEMED (*N*,*N*,*N*',*N*'-Tetramethylethylenediamine)	0.003

2.4.4 Gel electrophoresis

Gels were placed in a gel tank (Novex mini cell XCell II, Invitrogen, Paisley, UK) with running buffer (*Table 2-8*). Each well was loaded with a protein sample with a determined protein mass of 50 µg unless stated otherwise. Rainbow Marker (Precision Plus Protein™ Standards, Bio-Rad, Hemel Hempstead, UK) was loaded in to the farthest left well followed by a biotinylated marker to allow visualisation of protein movement. The gel was set to run at 200 V for approximately 50 min.

Table 2-8: Composition of the running buffer

Composition	Concentration (mM)
Trizma Base	24.8
Glycine	191.8
SDS	3.5

All components are purchased from Sigma Aldrich (Poole, UK).

2.4.5 Protein transfer

Blotting sponges were soaked in transfer buffer (*Table 2-9*) and air bubbles were removed by pressing the sponges with a glass pipette. The pattern of the blotting sandwich is shown in *Figure 2-4*; it was covered with transfer buffer whilst the main buffer chamber of the tank was filled with deionised water. Proteins from the gel were transferred onto Polyvinylidene Fluoride (PVDF) membrane (Hybond-P, Amersham, Buckinghamshire, UK) at 30 V for 2 h. The negatively charged protein then exited the gel, travelled towards the anode and bound to the membrane.

Table 2-9: Composition of the transfer buffer

Composition	Concentration (mM)
Trizma Base	19.8
Glycine	150.5
Methanol	20%

All components were purchased from Sigma Aldrich (Poole, UK).

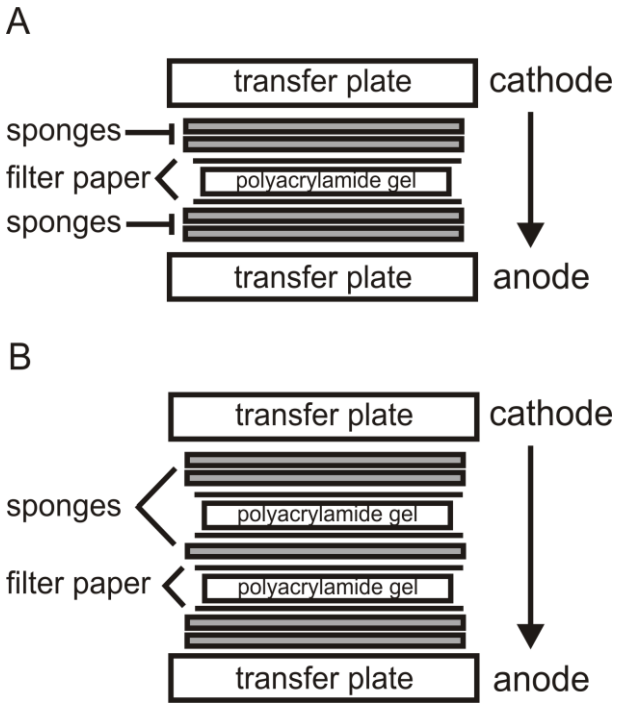

Figure 2-4: Typical pattern for sandwich blotting for protein transfer in a Western standard protocol for (A) one gel and (B) two gels

2.4.6 Immunodetection

After the transfer process, the PVDF membrane was incubated in TBS-T (8.8 g/l NaCl, 0.2 g/l KCl, 3.0 g/l Tris and 0.05% Tween-20 pH 7.5) with 2% non-fat dry milk (hereafter simply called "blocking buffer") for 1 h at room temperature. This was to reduce any nonspecific binding and to reduce background staining on the membrane. The membrane was incubated with the desired primary antibody at the optimum working dilution for blocking buffer, respectively (*Table 2-10*). All incubation was conducted overnight at 4^{o}C on a roller so that every part of the membrane was covered with the antibody solution. The next day, the membrane was washed with TBS-T (without milk) for 10 min. This was repeated three times then followed by incubation with the appropriate secondary antibody conjugated to Horse Radish Peroxidase (HRP) for ~1 h at room temperature along with gentle agitation. A further three washes with TBS-T for 10 min were then carried out to remove excess milk powder residue.

2.4.7 Chemiluminescence and film exposure

After the final wash with TBS-T, the buffer was discarded and the remaining buffer on the membrane was drained off. Enhanced chemiluminescence (ECL) reagent I and II were mixed in the appropriate amounts (*Table 2-11*) and the mixture was added to the membrane then incubated at room temperature for 1 min. The excess mixture of reagents was dried before being wrapped in clear plastic. All bubbles were gently removed by pressing the plastic with tissue. Once in the dark room under safe lighting the membrane was placed protein side up in an exposure cassette. A piece of film (Hyperfilm ECL, Amersham, Buckinghamshire, UK) was exposed for the required time for the respective antibody and developed using a Compact 4 automatic film developer (X-ograph imaging systems, Wiltshire, UK).

Table 2-10: Antibody application for Western analysis

Primary Antibody	Working dilution	Secondary Antibody	Working Dilution
Anti α-ENaC[1]	1 µg/ml	Rabbit anti mouse HRP conjugate	1: 3000
Anti β-ENaC[2]	2 µg/ml	Goat anti rabbit HRP conjugate	1: 3000
Anti γ-ENaC[3]	1 µg/ml	Goat anti rabbit HRP conjugate	1: 5000
Anti β-Actin[3]	2 µg/ml	Rabbit anti mouse HRP conjugate	1: 10,000
Anti Phospho [4]NDRG1-Thr[346/356/366]	1.5 µg/ml	Rabbit anti sheep HRP conjugate	1: 5000
Anti Total [4]NDRG1-Thr[346/356/366]	0.35µg/ml	Rabbit anti sheep HRP conjugate	1: 10,000
Anti Phospho [5]PKB-Ser[473]	1 µg/ml	Goat anti rabbit HRP conjugate	1: 3,000
Anti Phospho [5]PKB-Thr[308]	1 µg/ml	Goat anti rabbit HRP conjugate	1:2,000
Anti Total [5]PKB	1 µg/ml	Goat anti rabbit HRP conjugate	1:3,000
Anti Phospho [5]PRAS40-Thr [246]	0.3 µg/ml	Rabbit anti sheep HRP conjugate	1: 2,000
Anti Total [5]PRAS40	0.52µg/ml	Rabbit anti sheep HRP conjugate	1: 2,000
Anti Phospho [4]Nedd4-2-Ser [221]	5 µg/ml	Rabbit anti sheep HRP conjugate	1: 5,000
Anti Phospho [4]Nedd4-2-Ser [327]	2 µg/ml	Rabbit anti sheep HRP conjugate	1: 5,000
Anti Phospho [4]Nedd4-2-Thr [246]	2 µg/ml	Rabbit anti sheep HRP conjugate	1: 5,000
Anti [4]Nedd4-2	1 µg/ml	Rabbit anti sheep HRP conjugate	1:10,000
Anti [5]CREB-Ser[133]	1 µg/ml	Goat anti rabbit HRP conjugate	1: 1000
Anti [5]CREB	1 µg/ml	Goat anti rabbit HRP conjugate	1:1000

[1] - Gift from Cystic Fibrosis Centre, North Carolina University, US; [2] - Santa Cruz Biotechnology, Wembley, UK; [3] - Sigma Aldrich, Poole, UK; [4] - Gift from MRC Phosphorylation Protein Unit, University of Dundee, UK; [5] - Cell Signalling, Hertfordshire,UK

Table 2-11: Recipe for enhanced chemiluminescence (ECL) solutions

Components	Concentration (M)	Volume (ml)
Solution I		
Luminol	0.25	2.5
p-coumaric acid	0.9	1.1
Tris (pH 8.5)	1	25.0
Deionised water	-	221.4
Solution II		
H_20_2	0.3	0.16
Tris (pH8.5)	1	25.0
Deionised water	-	224.8

All components were purchased from Sigma Aldrich (Poole, UK)

2.5 Experimental design and statistical analysis

A densitometry analysis was performed on each band image on the developed film. Each film was scanned using a standard scanner and further analysed with the ImageJ 1.46 software (NIH; **http://imagej.nih.gov/ij/download.html**).

For α, β, and γ-ENaC proteins in both the total lysates and cell surface extraction, a raw value for the densitometry was taken as the sum of pixel values minus a background value for each pixel within a bounded area. The treatment value was determined by relative comparison to the control sample. A similar approach was applied to the remaining endogenous phosphorylated and total protein kinases individually. Results from at least 3 independent cell passages (strictly age-matched cells) and repetitions were presented as the mean ± standard error (SEM) and further analysed using a *Student's* paired t-test where $*p<0.05$, $**p<0.01$ and $***p<0.001$

represent a significant difference between the means. If there were more than two means involved in the analysis, one-way ANOVA and Bonferroni *post-hoc* tests (SPSS) were conducted; †$p<0.05$. Any values of n refer to independent passages in each group.

3

Epithelium sodium channels (ENaC) expression in H441 cells

3.1 Introduction

Fluid clearance from the lungs at birth is vital and is thought to be regulated by glucocorticoids (GCs) which are naturally found in blood. GCs are often used to treat cases of respiratory distress syndrome (RDS) and pulmonary oedema, as a lack of these hormones can contribute to poor fluid regulation during birth and disruption of lung function in adult life. Despite considerable evidence to support this theory, the mechanisms by which GCs act are still not fully understood.

To investigate GC-stimulated Na^+ transport in epithelial sodium channels (ENaC), the H441 human bronchiolar cell line was used as a model. ENaC proteins are present on the cell surface of the lung epithelial. ENaC is a multimeric protein that consists of three structurally related subunits: α-, β-and γ- (Cannessa *et al.*, 1996). It is a key component in the regulation of the rate of Na^+ transport. The channel exhibits a high selectivity for Na^+, and is amiloride-sensitive when expressed in *Xenopus laevis* oocytes or mammalian tissue. In the human distal lung epithelium of H441 cells, dexamethasone is known to stimulate amiloride sensitive Na^+ transport (Sayegh *et al.*, 1999; Ramminger *et al.*, 2004; Clunes *et al.*, 2004; Brown *et al.*, 2008; Greenwood *et al.*, 2009; Inglis *et al.*, 2009; Watt *et al.*, 2012) by increasing α-ENaC mRNA

expression (Sayegh *et al.*, 1999; McTavish *et al.*, 2009), and upregulating α- and β-ENaC (Ramminger *et al.*, 2004; Thomas *et al.*, 2004) and all ENaC subunits (Itani *et al.*, 2002b). Therefore, H441 cells are a useful model for investigating the activation of ENaC via GCs (dexamethasone), and they have been used for other physiological studies of Na^+ transport with similar hormones (Brown *et al.*, 2008; Gallacher *et al.*, 2009; Watt *et al.*, 2012).

The ENaC protein can be activated by α-ENaC alone or through dual-expression with either β- or γ-ENaC (Canessa *et al.*, 1994). The dual expression reflects the different properties of the heteromultimer (McNicholas & Canessa, 1997). This illustrates that ENaC can be constructed through different stoichiometry to be functional. The most convincing study to date suggests that expression of the α-subunit is the most critical, as a lack of this subunit results in an inability to clear lung fluid (Hummler *et al.*, 1996).

ENaC expression at the cell surface is thought to be regulated by glucocorticoid-regulated kinase 1 (SGK1) (Kamynina *et al.*, 2001a; Kamynina *et al.*, 2001b; Boyd & Fejes-Tóth, 2005; Lee *et al.*, 2007). SGK1 is known to stimulate epithelial sodium channel genes in the human foetal lung (Venkatesh & Katzberg, 1997). When SGK1 activity is increased, it augments ENaC trafficking towards the cell surface by phosphorylating the ubiquitin ligase protein, Nedd4-2 (Debonneville *et al.*, 2001; Snyder *et al.*, 2002; Kamynina & Staub, 2002). In normal cells, ENaC regulation is controlled through ubiquitination. Thus, when Nedd4-2 is inhibited by SGK1, it will no longer bind to ENaC (Debonneville *et al.*, 2001; Snyder *et al.*, 2002) and this allows more ENaC proteins to be expressed at the cell surface. An abundance of ENaC proteins will generate a higher G_{Na+} , causing an increase in Na^+ absorption (Paunescu *et al.*, 2000). However, the deletion of the SGK1 gene only causes mild effects towards renal Na^+ handling (Wulff *et al.*, 2002; Fejes-Tóth *et al.*, 2008; Lang *et al.*, 2009), as the viable mice do not display any functional abnormalities. Similar observation can also be seen in the colonic ENaC activity

(Rexhepaj *et al.*, 2006) which suggests that renal and colonic ENaC regulation may involve different mechanisms. It would be expected that lack of SGK1 gene is not the sole regulator in trafficking ENaC to the membrane.

Therefore, SGK1 activation needs to be investigated in order to clarify its role in ENaC expression. The SGK1 protein needs to be phosphorylated at two distinct residues in order to confer its activity (Biondi *et al.*, 2001), which is dependent on PI3K activity (Kobayashi & Cohen, 1999; Park *et al.*, 1999; Biondi *et al.*, 2001). However, there is limited information available on either SGK1 or PI3K activity in H441 cells or their response to GCs, compared with the number of studies on renal epithelia. Therefore, we chose to investigate SGK1 activity in the pulmonary epithelia using the H441 cell line. Our group uses transfected H441 cells with mutant forms of SGK1 and P13K that are either constitutively active or catalytically inactive and have observed a non selective conductance of Na^+ transport (Brown *et al.*, 2008; Inglis *et al.*, 2009). As yet, the activities of SGK1 and P13K have not been fully investigated in this cell line.

The protein kinase N-myc Downstream Regulated Gene 1 protein (NDRG1) is a good indicator for SGK1 activity as it is phosphorylated at $Thr^{346/356/366}$ by SGK1 and not by other related kinases such as PKB and S6K1 (Murray *et al.*, 2004). Antibodies raised against phosphorylated NDRG1-$Thr^{346/356/366}$ have been used to monitor SGK1 activity (Murray *et al.*, 2004) in HeLa cells and *sgk1* knockout mice. The same technique can be applied for observation of SGK1 activity in mouse cortical collecting duct cells (Mansley & Wilson, 2010a) and H441 cells (Inglis *et al.*, 2009; McTavish *et al.*, 2010).

3.2 Experimental design: aims and objectives

Since it corresponds to the activation of SGK1 (Murray *et al.*, 2004; Inglis *et al.*, 2009; Mansley & Wilson, 2010a; Watt *et al.*, 2012), the phosphorylation of NDRG1-Thr$^{346/356/366}$ was investigated first. Since dexamethasone is thought to elevate SGK1 activity, an experiment was carried out to determine the time and dosage-dependent incubation of dexamethasone on SGK1 activity. This study complements work done by Watt *et al.*, (2012), who noted an amiloride sensitive Na^+ current in cells treated with dexamethasone. Therefore, ENaC protein expression is a good way to study the rate of Na^+ transport. Many studies focusing on the physiological mechanisms underlying Na^+ transport have used cells cultured on a permeable membrane. However since different cell culture conditions may result in different ENaC protein expression, I designed an experiment that examined the expression of ENaC subunits on both a permeable support with an air-liquid interface (transwell) and on a hard surface (6-well or petri dish).

Incubation with dexamethasone (for ~24 h) has been shown to stimulate Na^+ transport in H441 cells due to amiloride sensitivity (Ramminger *et al.*, 2004; Clunes *et al.*, 2004; Brown *et al.*, 2008; Inglis *et al.*, 2009; Watt *et al.*, 2012). This increased Na^+ transport in GC-stimulated cells is correlated with an increased expression of ENaC protein at the cell surface suggesting that the two may be related. Therefore, in this section of the thesis I investigate ENaC protein expression in GC-stimulated H441 cells.

Previous studies have not always succeeded in successfully detecting ENaC subunit protein expression due to the low abundance of this protein. On the occasions that ENaC subunit expression was thought to be detected, the results were considered inconclusive due to the possible nonspecific protein binding of the antibodies. Therefore, at start of this project a wide selection of antibodies were used to observe the expression of each subunit in both the total

lysates and cell surface pool proteins. This procedure was optimised to select antibodies that specifically detected each subunit protein of ENaC, thereby minimising nonspecific protein binding. To further assess the specificity, ENaC protein expression was confirmed by monitoring the absence of each protein band following competition with immunogenic peptides where they were commercially available. The preliminary results then allowed me to identify the expression of each subunit of ENaC (α-, β-, and γ-) in H441 cells, in both the intracellular pool proteins and at the cell surface. In addition, I observed ENaC protein trafficking towards the cell surface in the presence of dexamethasone and compared this to trafficking in GC-deprived cells. The cell surface proteins were labelled with biotin to further probe for ENaC subunits. This method enabled us to successfully extract ENaC subunit protein from the cell surface, and compare it to the intracellular pool proteins which were indicated by the absence of β-actin, which acted as a marker for the purity of the biotin-labelled protein extracted from the cell surface.

In summary, the aims of this chapter are:

1) To observe SGK1 activity using the phosphorylated-NDRG1Thr$^{346/356/366}$ antibody in GC-induced H441 cells.
2) To identify ENaC protein expression in H441 cells.
3) To differentiate ENaC protein expression in H441 cells that is cultured on either a permeable or on a hard-surface plate.
4) To establish ENaC protein expression in GC-treated, as well as GC-deprived H441 cells, both intracellularly and on the cell surface.

3.3 Results

3.3.1 Dose-dependence of dexamethasone-treated H441 cells

Cells grown on 6-well plates were maintained in *complete media* until confluent. The media was then changed to a defined media but only supplemented with *dialysed serum* ~24 h prior to obtaining the cell lysates. During this period, dexamethasone of varying dosages (0, 10, 50, 100, 150 and 200 nM) was introduced to the cells in the final ~3 h. After Western analysis, the densitometry values of both the phosphorylation of NDRG1-Thr$^{346/356/366}$ and the total protein were analysed (GraphPad Prism) (*Figure 3-1A*). *Figure 3-1B* confirms the data previously found by our lab, as a dosage of 200 nM (or 0.2 µM) successfully evoked the expression of the SGK1 indicator (Ramminger *et al.*, 2004; Clunes *et al.*, 2004; Brown *et al.*, 2008; Inglis *et al.*, 2009; Watt *et al.*, 2012). A sigmoidal log curve that reaches a peak showing an almost 100% ratio over control cells that were treated with the maximum dosage also reconfirms data that was found previously in our lab (Mansley, 2010; Watt, 2011). The NDRG1 protein was loaded equally to eliminate any error when interpreting changes in protein phosphorylation (*Figure 3-1A*).

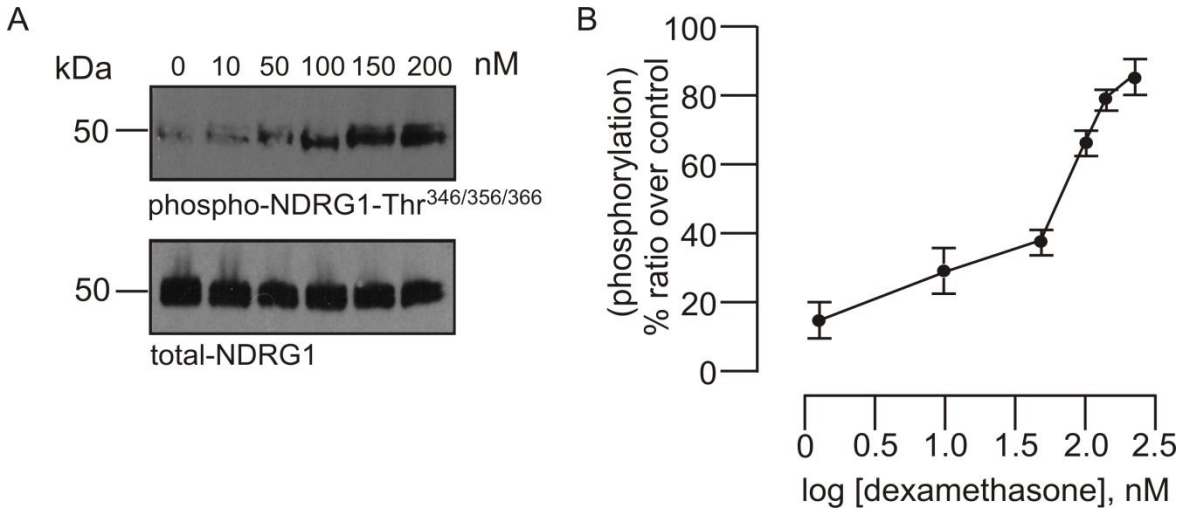

Figure 3-1: Dose-response of dexamethasone (nM) upon phosphorylation of NDRG1-Thr$^{346/356/366}$

(A) The densitometry of NDRG1-Thr$^{346/356/366}$ phosphorylation (upper blot) and total protein (lower blot) and their response to concentrations of dexamethasone (3 h) in a cumulative manner. (B) The sigmoidal graph represents the log of concentrations versus phosphorylation of NDRG1-Thr$^{346/356/366}$. Each point represents the mean ± SEM of 3 independent samples.

3.3.2 Time-dependence of dexamethasone treated H441 cells

Cells grown on 6-well plates were maintained in *complete media* until confluent. The media was then changed to a defined media but only supplemented with *dialysed serum* ~24 h prior to collection of the cell lysates. During this period, dexamethasone (0.2 µM) was introduced to the cells at a variety of points between 0 - 24 h. Similar groups of age-matched cells were not hormonally treated so that they could act as a control. All cells were later probed for NDRG1-Thr$^{346/356/366}$. Phosphorylation of this protein at Thr$^{346/356/366}$ increased due to the effects of dexamethasone (0.2 µM) at 1 h and reached maximal expression at 3 h (*Figure 3-2A,B*). The amount of NDRG1-Thr$^{346/356/366}$ decreased after this and continued to do so up to the 24 h point. The phosphorylated expression of NDRG1 did not affect the total protein abundance (*Figure 3-2B*).

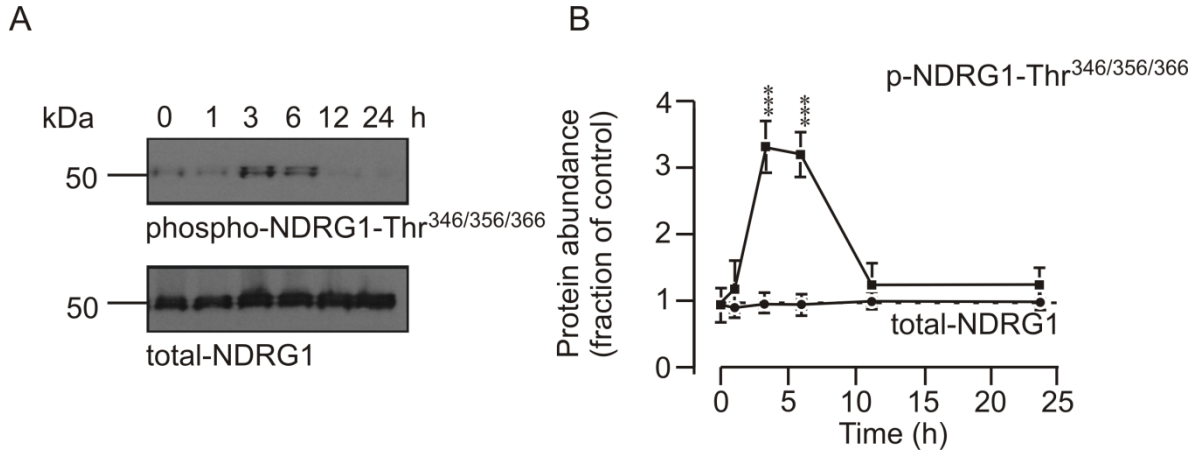

Figure 3-2: Effects of dexamethasone (0.2 µM) on NDRG1-Thr$^{346/356/366}$ phosphorylation and total protein over time

(A) A typical Western blot showing the effects of dexamethasone (0.2 µM) on NDRG1-Thr$^{346/356/366}$ phosphorylation and total NDRG1 starting from 0 min until ~24 h. (B) The graph shows the densitometry values for the protein abundance in (A). Data represents the mean ± SEM (n = 4). Asterisks denote a significant difference towards dexamethasone-deprived cells; *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA, Bonferroni *post-hoc* test).

3.3.3 Immunogenic peptides of ENaC

The expression of ENaC subunits was confirmed by introducing immunogenic peptides raised against the antibody. This was crucial to confirm that the protein bands obtained from the protein lysates were specific, as the molecular weight suggested. *Figure 3.3* illustrates the indicative molecular weight of (A) β-ENaC; ~100 kDa and (B) γ-ENaC (~90 kDa and ~75 kDa) (left panels) and when they were neutralised after incubation with immunogenic peptides (right panels). Expression of α-ENaC could not be performed, as the α-ENaC antibody was a gift from North Carolina University, and no commercial immunogenic peptides have been raised to this antibody to confirm the molecular weight. Nevertheless, an approximate molecular weight of between 95kDa and 75kDa for α-ENaC protein has been shown by other studies (*Table 3-1*).

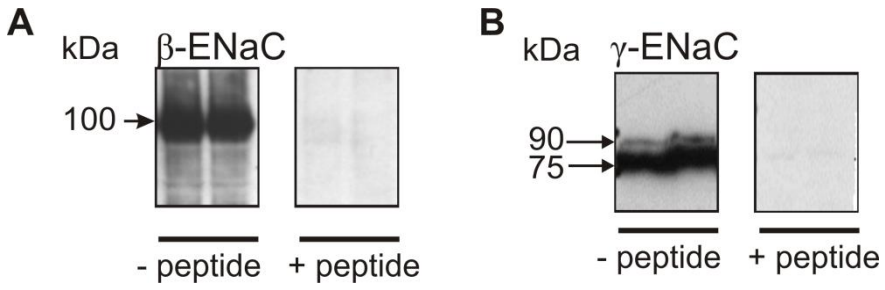

Figure 3-3: The validation of ENaC protein

(A) Inhibitory peptides for the β-ENaC antibody were raised against the C-terminus. The peptide successfully inhibited the protein at ~100 kDa. (B) Inhibitory peptides for the γ-ENaC antibody were raised against the N-terminus. The peptide inhibits both isoforms of the protein at ~90 kDa and ~75 kDa.

Table 3-1: Molecular weight of ENaC in various cell types

Cell Surface Bionylation of ENaC		α-ENaC		β-ENaC	γ-ENaC	
Authors	**Cell type**	**Full Length**	**Short Length**		**Full Length**	**Short Length**
Planés *et al.*, 2002	ATII	85 kDa	65 kDa			
Booth & Stockand, 2003	A6	90 kDa		95 kDa	95 kDa	70 kDa
Awayda *et al.*, 2004	oesophageal	90 kDa	75 kDa	105 kDa	90 kDa	
Myerburg *et al.*, 2006	HAEC	97 kDa	75 kDa			
Mace *et al.*, 2008	H441	90 kDa	65 kDa	100 kDa	90 kDa	
Woollhead & Baines, 2006	H441	93 kDa				
Xu & Chu, 2007	H441	85 kDa	70 kDa		80 kDa	
Knight *et al.*, 2006	HEK 293T	90 kDa	65 kDa		85 kDa	75 kDa
Raikwar &Thomas, 2008	HEK293T		75 kDa			
Ruffiex Daidie *et al.*, 2008	HEK293T	110 kDa	90 kDa	95 kDa	77 kDa	73 kDa
Zhou *et al.*, 2007	HEK293T	90 kDa	65 kDa	100 kDa		
Kim *et al.*, 2007	mature kidney mice	85 kDa		85 kDa	85 kDa	70 kDa
Randrianason *et al.*, 2007	mature lung transgenic mice	85 kDa	65 kDa	95 kDa	90 kDa	75 kDa
Hanwell *et al.*, 2002	MDCK (FLAG-AB)	100 kDa	80 kDa	110 kDa		75 kDa
Butterworth *et al.*, 2005	mpkCCDc14	95 kDa		90 kDa	75 kDa	65 kDa
Hughey & Kleyman, 2007	MDCK				85 kDa	70kDa
Hughey *et al.*,2004	MDCK	96 kDa	65 kDa	110 kDa	95 kDa	75 kDa
Vasquez *et al.*, 2008	SMG-C6	90 kDa				
Diakov *et al.*, 2008	*X.laevis* Oocytes				87 kDa	76 kDa (total) 67 kDa (surface)
Hu *et al.*, 2009	*X.laevis* Oocytes	80-85 kDa	60-65 kDa			
Harris *et al.*, 2007	*X.laevis* Oocytes	80 kDa	65 kDa	105 kDa	87 kDa	76kDa
Malik *et al.*, 2005	*X.laevis* Oocytes	86 kDa				
Bruns *et al.*, 2007	*X.laevis* Oocytes	95 kDa	65 kDa		87 kDa	75 kDa

3.3.4 ENaC expression in different growth substances

H441 cells were cultured in *complete media* prior to confluence. The media was then changed to a defined media for a further ~ 24 h. At this point, cells were treated with dexamethasone (0.2 µM) for 24h. H441 cells were cultured on two different substances: a 6-well plate and a porous membrane (transwell). All treatments were added to the basal side of the transwell, and to the medium in the 6-well plate, before they were extracted and added to the SDS gel. Two bands that correspond to α-ENaC (~95 kDa and ~75 kDa) were consistently detected, a single band was seen for β-ENaC (~100 kDa) and the two isoforms of γ-ENaC (~90 kDa and ~75 kDa) were detected. This indicated that each antibody was able to probe successfully (*Figure 3-4A*).

Figure 3-4A shows the results for the H441 cells cultured on both 6-well plates and porous membranes. Cells treated with dexamethasone showed increased expression of α- and β-ENaC but not γ-ENaC. This was demonstrated by cells cultured both on the hard surface and the porous membrane. This observation is in contrast to that of Zucchi *et al.*, (2002), who reported that cells that were cultured on a membrane created a dome-like formation that increased the abundance of β-ENaC. Although cells cultured on the membrane contribute to an increased G_{Na+} polarisation and transepithelial resistance (Inglis *et al.*, 2009), ENaC expression is similar for cells cultured on either a hard-surface or a membrane. However, since H441 dexamethasone-treated cells cultured on a hard surface can be hyperpolarised and display an amiloride sensitive current (Watt *et al.*, 2012), I decided to continue to culture the cells on a hard-surface (6-well plate) in subsequent experiments.

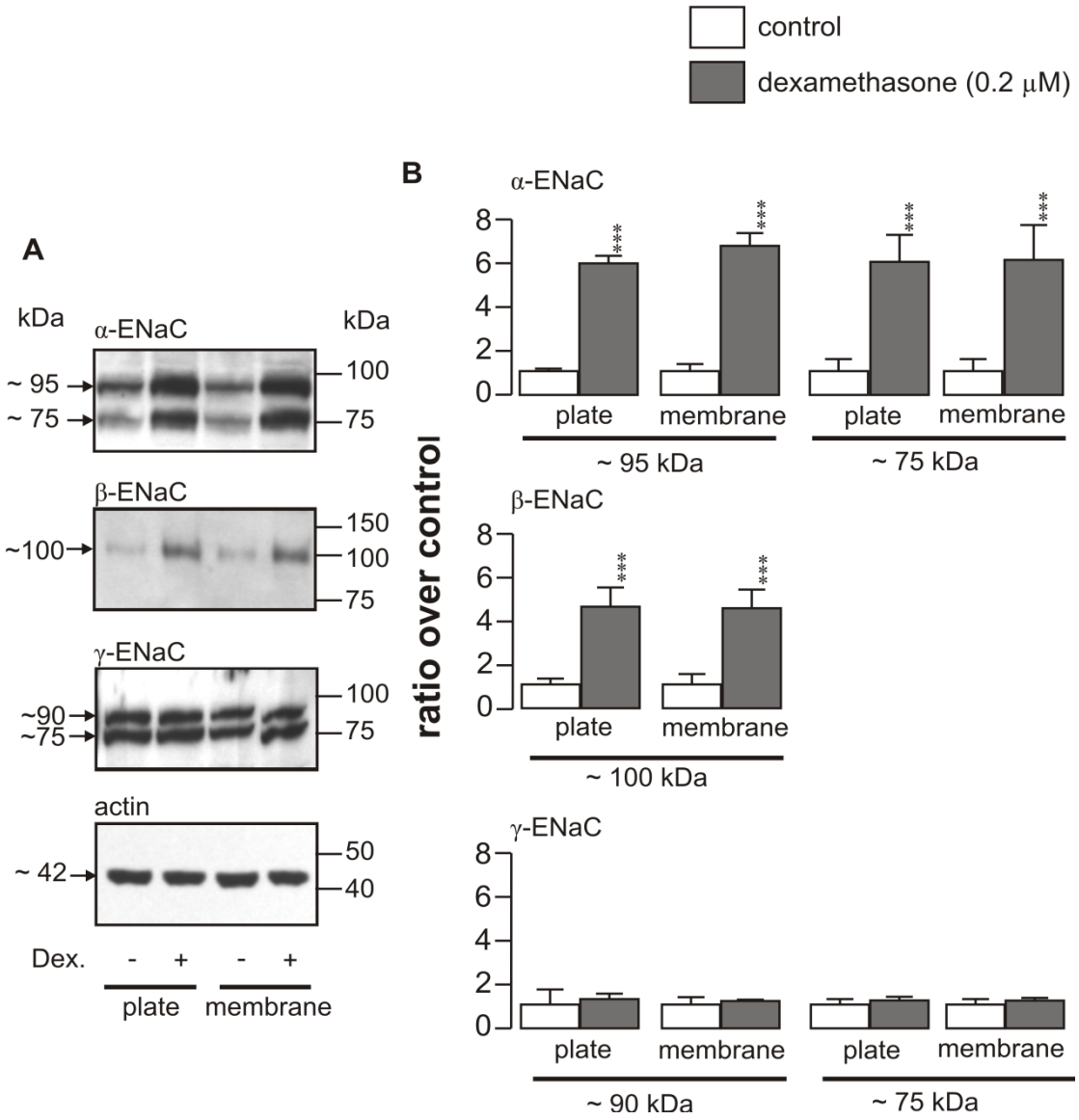

Figure 3-4: The expression of ENaC (α-, β-, γ-) in the presence of dexamethasone (0.2μM) in H441 cells grown on different substances

(A) Western blots showing the expression of the α-, β-, γ- subunits of ENaC in H441 cells raised on two different substances; a 6-well plate and a 6-well plate containing a porous membrane (Transwell, Corning Star). The cells were maintained until confluent for ~5 days in a medium supplemented with insulin. They were then changed to a fully defined medium with dexamethasone, Dex. (0.2 μM) for ~ 24 h prior to the collection of lysates. The experiment is representative of four independent samples. (B) Densitometry analyses of blots (mean ± SEM) as shown in (A). Treatment with dexamethasone (0.2 μM) for ~24 h showed a significant difference compared to control cells (glucocorticoids-deprived) (paired *Student's* t-test, ***p<0.001, n = 4).

3.3.5 Cell surface biotinylation – the access of actin

The cell surface of H441 cells was labelled with biotin and then bound to streptavidin for Western analysis (for a detailed methodology see *Chapter 2*; **2.3.2**). The surface protein that was extracted using this method was initially probed for actin; the absence of actin confirms a pure extraction from the surface pool proteins and shows if there has been any leakage from the intracellular space. To check for such a contamination, cells derived from the biotin-streptavidin bound cell surface were analysed using the RC-DC assay and then subjected to Western analysis. Eight independent experiments revealed that 48 ± 0.68 µg of protein could be recovered from an initial 500 µg of total lysates. An aliquot of 1µg of cell lysate served a positive control to compare the intensity of different protein amounts derived from the surface pool. *Figure 3-5A* shows the 30 minute over-exposure blot from the 1µg of intracellular protein from the H441 cells, along with 5 µg, 10 µg and 20 µg from the cell surface abundance. *Figure 3-5B* shows the fraction of total lysates and reveals a significant difference compared to the cell surface abundance of ENaC. The presence of β-actin in the biotinylated protein fraction shows that this protein pool does contain some intracellular proteins. Hence, the densitometric analysis shows that the abundance of β-actin in a 20 µg aliquot of biotinylated protein was ~30% of the abundance measured in 1 µg of the total protein. The biotinylation-streptavidin binding protocol therefore allows surface-exposed proteins to be isolated with 98 – 99% purity.

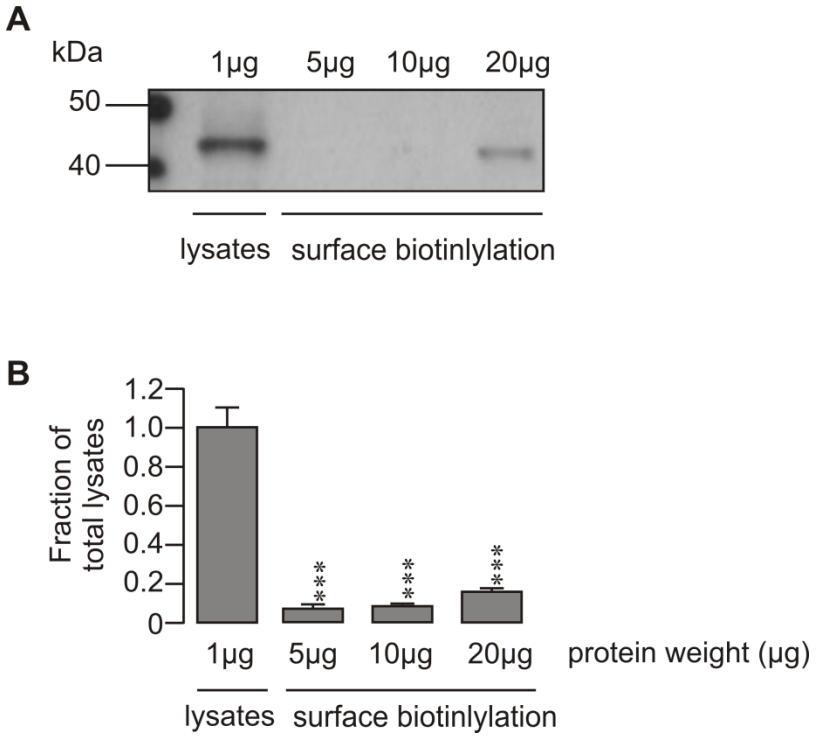

Figure 3-5: Actin purity in overexposure of 30 minutes

(A) A typical Western blot showing the over-expression of actin in the total lysate (1µg) and cell surface extraction (5 µg, 10 µg, 20 µg, respectively) in 30 minutes. (B) Densitometric analysis of the data from (A) showing the ratio of the difference in mean abundance on the cell surface compared to total lysates. Data are presented as the mean ± SEM (n = 8). Asterisks denote statistical significance when compared to the initial values (one-way ANOVA, Bonferroni *post-hoc* test; ***p<0.001).

3.3.6 The expression of ENaC subunits intracellularly and on the cell surface

H441 cells were grown on a 6-well plate, treated with glucocorticoid-deprived media and placed in a media supplemented with dexamethasone (0.2 µM) for ~ 24 h. The cells were then labelled on ice with biotin-streptavidin and centrifuged (14,000 x *g*) to extract the cells from the surface abundance, then fractioned using Western analysis with samples that were not labelled with streptavidin (intracellular lysates). These proteins were then subsequently probed for α-, β-, and γ-ENaC.

Figure 3-6A depicts the expression of each subunit of ENaC (α-, β-, and γ-) in two different pools of proteins. The absence of β-actin in the cell surface pool proteins confirms that these subunits are purely biotinylated and extracted from the surface abundance. As anticipated, α-ENaC was present in two isoforms, ~ 95 kDa and ~ 75 kDa, in both pools of proteins. A single band of β-ENaC (~ 100 kDa) was expressed in both the total lysates and the surface abundance. Interestingly, γ-ENaC expression was observed as a single band (~75kDa) from the surface abundance as opposed to two isoforms (~90 kDa, ~75kDa) as shown by the intracellular pool of proteins. As a housekeeping control, these blots were stripped with acid-stripped buffer (pH 2.2) and reprobed for actin in order to confirm its presence in the intracellular protein pool and its absence from the surface abundance pool proteins.

The bar graphs in *Figure 3-6B* represent the effects of dexamethasone compared to the control cells. As seen previously, dexamethasone enhances the expression of both isoforms of α-ENaC in the total lysates. The effect is similar for the surface protein. However, it only increases the abundance of β-ENaC in the total lysates, not on the cell surface. Interestingly, dexamethasone does not affect the abundance of γ-ENaC isoforms in the intracellular of pool proteins, but significantly promotes the expression of the smaller form of the subunit on the endogenous cell surface.

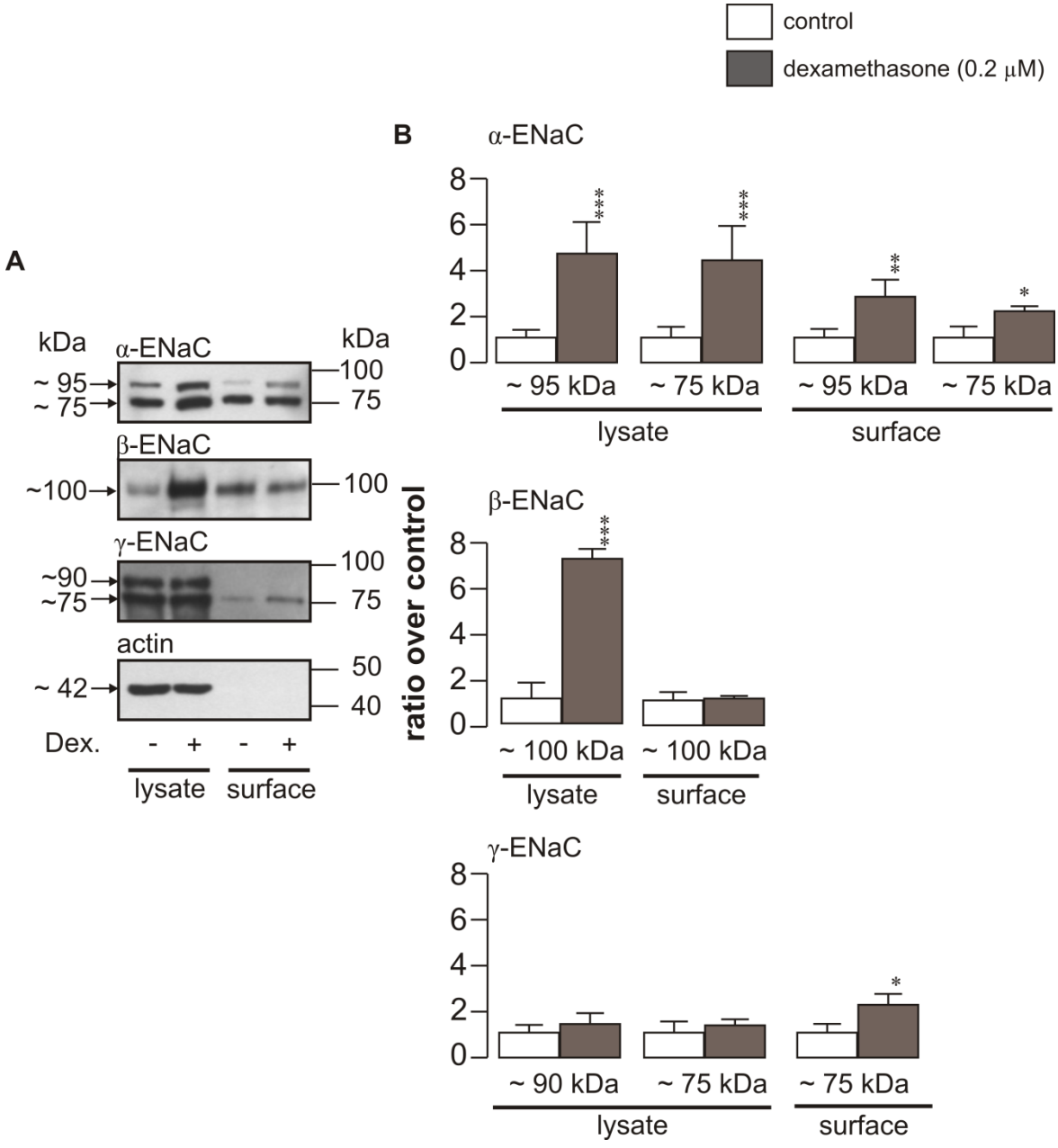

Figure 3-6: The trafficking of ENaC subunits (α-, β-, γ-) from the total lysates towards the cell surface of H441 cells.

(A) Western blots showing the expression of the α-, β-, and γ- subunits of ENaC derived from a different location, total lysates and protein derived from cell surface biotinylation. Actin expression was also quantified in order to ensure that the isolation of cell surface protein extraction was successful. Each blot represents 4 independent experiments. (B) Densitometry analyses comparing the effect of dexamethasone, Dex. (0.2 μM) upon the abundance of ENaC subunits in both the total lysates and the cell surface (n = 4, *Student's* t-test *p<0.05, **p<0.01, ***p<0.001).

3.4 Discussion

3.4.1 Dexamethasone increases SGK1 activity

Until recently, direct assays of SGK1 protein activity have not been successful due to the difficulty in detecting SGK1 protein expression. However, by assaying for NDRG1 – $Thr^{346/356/366}$ and using it to track SGK1 activity previous studies in this lab have managed to indirectly assess the abundance of the phosphorylated form of the SGK1 target protein in the mouse collecting duct of kidney cells (Mansley & Wilson, 2010a) and the human distal airway cells, H441 (Inglis *et al.*, 2009; Watt *et al.*,, 2012). NDRG1–$Thr^{346/356/366}$ was initially discovered as a stress stimuli-induced gene by Kokame *et al.*, (1996) and was further identified by Murray *et al.*, (2004). The 43 kDa protein is rapidly phosphorylated by SGK1 but poorly regulated by any other protein kinases. The antibody used in this thesis recognised 3 phospho-threonine residues ($Thr^{346/356/366}$) that are specific to NDRG1 but not the other two sites; Thr^{328} and Ser^{330} which are conserved in NDRG family members. Phosphorylation of the three afore mentioned residues showed a direct increase in SGK1 activity.

Until recently, there has been no other protein indicator that was suitable for reflecting cellular SGK1 activity as simply as NDRG1. SGK1 activity can be monitored through the phosphorylation of NDRG1-$Thr^{346/356/366}$ which has been shown to affect SGK1 activation, but not other related kinases (Murray *et al.*, 2005; Inglis *et al.*, 2009; Mansley & Wilson, 2010 a,b; Watt *et al.*, 2012). Dexamethasone has been shown to acutely evoke NDRG1 phosphorylation, which corresponds to an increase in SGK1 mRNA in the Na^+ absorbing epithelia (Wang *et al.*, 2001; Itani *et al.*, 2002b; Thomas *et al.*, 2004). The maximal response to dexamethasone at 3-6 h indicates that hormonal stimulation promotes ENaC function, whereas prolonged exposure results in a return to the basal expression. It is only after this period that the amiloride sensitivity that is associated with ENaC activity develops

(Watt *et al.*, 2012). However, the data presented here reveal a reduction in expression after 3-6 h, though minor traces of phosphorylated protein can still be found. Considering these results, I propose that SGK1 transiently increases ENaC expression at the cell surface when it is active. Over time, when SGK1 activity is low, expression of α-ENaC in both pool proteins occurs, suggesting that SGK1 is not involved upon α-ENaC trafficking to the membrane. This must be assisted by an unknown mechanism, perhaps by an independent activation of α-subunit of the SGK1. The afore mentioned hypothesis supports a study by Zhang *et al.*, (2007) which showed that deletion of the *sgk1* gene resulted in only modest inhibition of the α-ENaC mRNA in the kidneys of mice fed on a low Na^+ diet, and showed no overt pulmonary phenotype (Wulff *et al.*, 2002).

3.4.2 ENaC expression on different substrates

A number of studies have suggested that H441 cells that are cultured on a porous membrane are better for investigating ENaC expression, as the confluent H441 monolayer cells effectively mimic the thin film of the alveolar distal epithelial lung cells (Sayegh *et al.*, 1999; Lazrak & Matalon, 2003; Thomas *et al.*, 2004; Ramminger *et al.*, 2004; Shlyonsky *et al.*, 2005; Inglis *et al.*, 2009). Dexamethasone has been shown to evoke apical Na^+ conductance and transepithelial resistance under these conditions, which corresponds well with the increased abundance of ENaC subunit mRNA (Itani *et al.*, 2002b). In theory, the increased levels of ENaC mRNA lead to higher expression of ENaC protein. However, this study has shown that the level of ENaC expression in H441 cells cultured on a permeable membrane is not significantly different to that of cells grown on a hard surface. This indicates that the substrate does affect the endogenous expression of ENaC. Although cells cultured on permeable membrane have previously been shown to form dome-structures (Zucchi *et al.*, 2001; Shlyonsky *et al.*, 2005) through the increase β-ENaC expression on the cell surface; this was not observed here.

Previous studies have shown that H441 cells that are cultured on a porous membrane are more likely to induce highly selective Na^+ absorption (Ramminger *et al.*, 2004; Inglis *et al.*, 2009) and demonstrate a non-selective Na^+ conductance (Albert *et al.*, 2008). Similarly, single H441 cells cultured on a coverslip and then subjected to the patching method (Brown *et al.*, 2008) did not display an amiloride-sensitive Na^+ conductance in the presence of dexamethasone. However, when a small group of 3-5 cells were cultured (prompted by a suggestion made by Zucchi *et al.*, (1999)) and the β-ENaC-related gene 133 was activated, the cells touched in order to generate an amiloride sensitive Na^+ absorption (Shlyonsky *et al.*, 2005; Brown *et al.*, 2008). Therefore, cell-cell communication is crucial for the amiloride sensitive phenotype by expressing tight junctions and other markers for β-ENaC. If this is true, then H441 cells that are cultured on a hard-surface may show a similar result, as they contribute to amiloride sensitive Na^+ conductance through a patch clamp experiments (Clunes *et al.*, 2004; Brown *et al.*, 2008; Watt *et al.*, 2012). Although the data in this chapter revealed that ENaC expression was similar under both conditions, I elected to grow the cells on a hard surface to make sure that cells were grown under similar conditions as those used in parallel patching studies.

3.4.3 ENaC trafficking towards the cell surface

ENaC is a complex protein that is expressed on the surface of epithelial cells. Theoretically, the protein requires the co-expression of three homologous proteins to form a functional multimeric channel. The expression of ENaC is thought to be elevated prior to the maximal Na^+ activity which appears to be initiated in the early phase of protein biosynthesis. ENaC trafficking involves protein maturation in the Golgi complex, with glycan processing at the N-terminus as it proceeds towards the cell surface (Rotin *et al.*, 2001; Ergonul *et al.*, 2006). Prior to activation, each subunit needs to be processed (through glycosylation or cleavage) before interacting to form a complex. The N-terminus of each subunit is responsible for this

process (Bruns *et al.*, 2003). Only α-ENaC (Ahn *et al.*, 1999) and γ-ENaC (Adam *et al.*, 1997) are thought to be involved in channel assembly, though Chalfant and colleagues have suggested that all N-termini of ENaC subunits contain an endocytic motif that allows each subunit to contribute to the construction of this channel, and therefore functionality at the cell surface (Chalfant *et al.*, 1999). Other studies have found that ENaC can bypass this process altogether (Hughey *et al.*, 2004b; Yu *et al.*, 2008). This is called the 'immature protein' (or full length) and it has been observed in this project. The antibodies for α- and γ-ENaC have been raised against the N-termini and the molecular weights have been noted prior to activation; as reviewed by Rossier and Stutts (2009).

Each individual subunit is expressed constitutively and was individually probed from two pools of proteins; the total lysates and the abundance of cell surface ENaC. There were similar expression levels for both α- and β-ENaC in both protein pools, however, the γ-ENaC from the surface pool appeared as a single band unlike its counterpart from the total lysate protein. In certain cases, the γ-ENaC from the surface protein was also seen to migrate more slowly than the predicted 75 kDa mass. This discrepancy indicates that the unglycosylated γ-ENaC may be involved in post-translation prior to trafficking towards the cell surface and is therefore restricted to the intracellular compartment.

Once ENaC has been delivered to the cell surface, each subunit can be cleaved, and thus activated, by serine proteases near the plasma membrane (Rossier & Stutts, 2009). This activation seems to be preserved in cells that express αγ-ENaC, but not αβ-ENaC. Therefore, the α-and γ-subunits of ENaC appear to be essential for channel activation by the extracellular proteases. This concurs with Hughey *et al.*, (2003), who suggested that both α- and γ-ENaC are cleaved prior to activation at the plasma membrane in kidney cells. They observed cleavage of α-ENaC twice and γ-ENaC only once in the presence of extracellular

furin protease. However, the data in this thesis suggests that α-ENaC and γ-ENaC were only cleaved into two isoforms in the total pool of proteins, and then trafficked towards the cell surface. Other products of the cleavage may not have been noticed, as the amounts of protein were too small to be detected through Western analysis. The two isoforms of α-ENaC remain at the cell surface for a longer period than the full length γ-ENaC, which disappeared due to degradation (Staub *et al.*, 1997a) and was confined to the intracellular compartment (Rossier & Stutts, 2009). Irrespective of this, β-ENaC remained uncleaved as reported by most other studies (refer to *Table 3-1*). This may be due to a lack of glycosylation sites (Harris *et al.*, 2007), and it may also act as a pre-dominant signal for channel removal (Kabra *et al.*, 2008).

3.4.4 Dexamethasone induced changes in ENaC expression

Dexamethasone-treated H441 cells increase ENaC protein expression. In the total lysates, this hormone induced the expression of α- and β-ENaC, which in turn was a consequence of increased levels of encoded mRNA (Ramminger *et al.*, 2004; Thomas *et al.*, 2004). Whilst all subunits were present in both pools of proteins, the synthetic GCs hormone only affected the abundance of α-ENaC. The GC effect is thought to be dependent on GC-response elements (GRE) in the α-ENaC gene (Itani *et al.*, 2002b; Thomas & Itani, 2004; McTavish *et al.*, 2009). Since activation of α-ENaC continues even after 24 h, this provides a mechanism by which amiloride-sensitive ENaC activity can be stimulated, indicating that the amiloride binding domain is within the α-subunit (Ismailov *et al.*, 1997; Kieber-Emmons *et al.*, 1999; Kashlan *et al.*, 2005). This finding is in contrast to previous studies which suggested that the whole magnitude of the Na^+ current is driven by increasing each subunit at the cell surface (Blazer-Yost *et al.*, 1998; Record *et al.*, 1998; Alvarez de la Rosa *et al.*, 1999; Paunescu *et al.*, 2000; Debonneville *et al.*, 2001; Alvarez De La Rosa & Canessa, 2003; Blazer-Yost *et al.*, 2003). However, though both types of pool proteins can

be dependent on α-ENaC alone, the highly functional channels may require a combination of β-ENaC and γ-ENaC (Jain *et al.*, 1999; Lazrak *et al.*, 2000; Jain *et al.*, 2001) for maximal activity.

In addition to the wide range of studies mentioned above, a collaboration carried out with Watt *et al.*, (2012) suggested that the amiloride sensitive Na^+ current is affected by the whole expression of α-ENaC in both pools of proteins as well as the role of γ-ENaC in the surface pool of proteins. This corresponds well with the Na^+ activity increment in dexamethasone-treated H441 cells (Lazrak & Matalon, 2003; Clunes *et al.*, 2004; Ramminger *et al.*, 2004; Thomas *et al.*, 2004; Shlyonsky *et al.*, 2005; Brown *et al.*, 2008; Inglis *et al.*, 2009). Since ENaC protein expression coincides with SGK1 activation, it suggests that the activity of SGK1 and ENaC may be correlated, especially in surface pool proteins. Therefore, in the next chapter I explore the role of SGK1 through the activation of PI3K and therefore the potential activation of ENaC protein expression in GC-treated H441 cells.

4

Role of P13-kinase and SGK1 in glucocorticoid-induced ENaC expression

4.1 Introduction

The results presented in the previous chapter show a role for dexamethasone, which appears to regulate the endogenous α-ENaC in both the total lysates and at the cell surface of H441 human airway cells. This data corroborates studies that reported that α-ENaC can both constitutively increase the natriferic response in the epithelial cells (Lin *et al.*, 1999; Otulakowski *et al.*, 1999; Sayegh *et al.*, 1999; Mick *et al.*, 2001; Diakov & Korbmacher, 2004) and be mediated by the SGK1 pathway upon the induction and maintenance of the Na^+ absorbing phenotype of the lung epithelium. Therefore, the absence of SGK1 would result in a failure to clear lung fluid at birth. However, the role of SGK1 is unclear, as there is no discernible lung phenotype in *sgk1* gene knockout mice: both histology and function are unaffected (Wulff *et al.*, 2002). This finding by Wulff and colleagues prompted us to investigate the role of the SGK1 pathway in ENaC expression on the cell surface of H441 cells. There is some evidence to indicate that PI3K may work independently of SGK1 to

induce Na^+ transport, as PKB isoforms that are down regulated by PI3K can mediate the effect of insulin on ENaC in rat thyroid cells (Lee *et al.*, 2007). This study further suggested that both SGK1 and PKB activity were required to maintain Na^+ basal transport, as siRNA directed against PKB and SGK1 has been shown to reduce amiloride sensitive Na^+ transport. This was even more successful in silencing both kinases when they were transfected together (Lee *et al.*, 2007).

There is some evidence to suggest that other kinases play a role in regulating ENaC. PI3K may affect ENaC through its second messengers PIP_2 and PIP_3, which have been shown to be involved in ENaC trafficking (Blazer-Yost *et al.*, 2004) and thus affect the channel gating kinetics (Pochynyuk *et al.*, 2007). Whilst the mechanism occurs transiently, a chronic effect has not been observed, leading to the conclusion that PIP_2 is required for constitutive ENaC activity that can be altered via other modulators (Pochynyuk *et al.*, 2007). Thus, PI3K activity could regulate ENaC independently of SGK1. However, as the cells were maintained in media containing corticosteroids, this possibility of PI3K acting alone remains experimentally unverified.

There are studies that have further explored the role of PI3K in Na^+ transport in H441 cells (Brown *et al.*, 2008, Gallagher, 2009; Inglis *et al.*, 2009). A constitutively active PI3K subunit (CD2-p110α) was transfected into the H441 cells in both GC-deprived and GC induced media. The transfection induced Na^+ transport in the presence of dexamethasone but not in the GC-deprived cells. This suggests that PI3K activity alone is not sufficient to induce Na^+ transport in single H441 cells (Brown *et al.*, 2008; Gallagher, 2009) but that it must be linked to SGK1 activity somehow. Nevertheless, PI3K is thought to be important because LY294002, a PI3K inhibitor, completely abolishes Na^+ transport in H441 cells (Gallagher, 2009; Inglis *et al.*, 2009). Recently, a more potent and specific PI3K inhibitor,

PI-103 (Bain *et al.*, 2007) caused a complete inactivation of PI3K but not SGK1 when monitored using NDRG1 phosphorylation (McTavish *et al.*, 2009), suggesting that SGK1 activity may not be fully dependent on PI3K.

All of the evidence above was taken into consideration when studying ENaC expression in the H441 cells. PI3K is thought to activate SGK1 through a signalling pathway involving TORC2 (Garcia-Martinez & Alessi, 2008; Lu *et al.*, 2010). TORC2 is responsible for the hydrophobic motif phosphorylation of PKB-Ser^{473} (Sarbassov *et al.*, 2005) therefore it can be used as an indicator for both PI3K activity and TORC2 (Thoreen *et al.*, 2009; Lu *et al.*, 2010; Mansley & Wilson, 2010a; Kuehn *et al.*, 2011). The phosphorylation of PKB-Ser^{473} is thought to enhance PDK1's dependence on the phosphorylation of PKB-Thr^{308} (Yang *et al.*, 2002; Sarbassov *et al.*, 2005), possibly by providing a docking site for PDK1. As PDK1 is dependent on PI3K activity, PKB-Thr^{308} phosphorylation can therefore be used to determine PDK1/PI3K activity. By blotting both PKB-Ser^{473} and PKB-Thr^{308}, PI3K activity can be monitored to allow a distinct inhibition between TORC2 and PDK1. However, care must be taken when interpreting the results, as PKB-Ser^{473} phosphorylation affects PDK1 dependent phosphorylation of PKB-Thr^{308} (Sarbassov *et al.*, 2005; Lu *et al.*, 2010). Therefore, in order to tackle this problem, the experiment was designed to monitor the effect of PI-103 on PI3K activity in both GC-induced and GC deprived cells, prior to determining whether PI-103 truly affects PI3K.

As described in the previous chapter, dexamethasone successfully evokes SGK1 activity through NDRG1-Thr$^{346/356/366}$. Recent evidence has suggested that TORC2 phosphorylates the hydrophobic motif of SGK1 (Lu *et al.*, 2010) through a docking site of PDK1, thereby promoting interaction between SGK1 and PDK1, which would cause phosphorylation of the activation loop of SGK1 at Thr^{256} to confer its activity (Biondi *et al.*, 2001). By introducing

a novel molecule, TORIN1, as a TORC2 inhibitor (Thoreen *et al.*, 2009), we can investigate the mechanism by which TORC2 stimulates SGK1 activity. As the inhibition is specific, it may provide an effective way to inactivate these kinases without causing inhibition of other closely related kinases involved in the proposed signalling pathway (Sherk *et al.*, 2008). In order to test the effects of TORIN1 on P13K activity, PKB-Thr^{308} phosphorylation was monitored. However, the problem with this approach is that TORC2 is responsible for PKB-Ser^{473} phosphorylation which in turn facilitates the phosphorylation of the Thr^{308} site (Andjelkovic *et al.*, 1997). This issue has been addressed by Thoreen and colleagues, who developed the inhibitor at a low concentration so that it only inhibits TORC2 at 0.1 µM (Thoreen *et al.*, 2009). Therefore, in this study I also used this concentration to inhibit TORC2 but not PDK1. In order to determine if this compound was specific to TORC2, I wanted to observe the expression of PRAS40 (proline-rich Akt/PKB substrate of 40 kDa) at Ser^{246}, a protein indicator that marks the activity of the PKB protein but no other protein kinases (Wullschleger *et al.*, 2006; Guertin & Sabatini, 2007). In addition, TORC2 activity can be observed by introducing rapamycin, an inhibitor that specifically hinders TORC1 activity but not TORC2. The data from Watt (2011) reported that rapamycin did not affect PI3K, SGK1 or ENaC activity, thus any effects of TORIN1 arise as a result of TORC2 inhibition. The effect of rapamycin is demonstrated by the de-phosphorylation of the ribosomal S6 kinase (S70-S6K) at Thr^{389}, a kinase that specifically confers TORC1 activity (Proud, 2007).

The lack of a specific inhibitor of SGK1 can be addressed by introducing a small molecule inhibitor, GSK650394. This was specifically developed by Sherk and colleagues (2008), who demonstrated that this inhibitor was able to block the enzyme activity of both SGK1 and SGK2 in scintillation proximity assays. This corresponded with the inhibition of an aldosterone induced amiloride-sensitive current in mouse cortical collecting duct cells (M1

cell line). By using an *in vitro* kinase assay, they were able to determine that GSK650394 was selective for SGK1 over PKB, PDK1 and other related kinases (Sherk *et al.*, 2008). Therefore, this novel compound can hinder SGK1 activity without affecting PI3K, PKB and PDK1. This confers the purpose of this study by inhibiting SGK1-related pathway (Sherk *et al.*, (2008); Lang *et al.*, (2009); Lang & Gorlach, (2010); and Mansley & Wilson, (2010a)) to further look its role in ENaC trafficking to the cell surface.

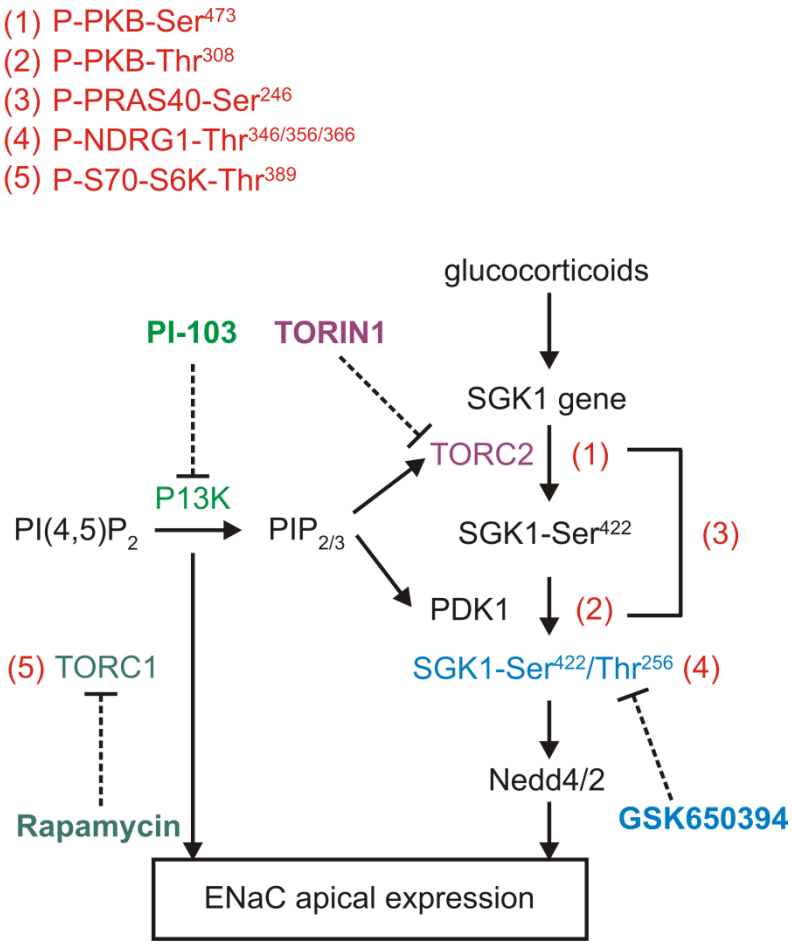

Figure 4-1: Diagram of the proposed signalling pathway for ENaC regulation.

All of the information that relates to the PI3K/SGK1 pathway is summarised in *Figure 4-1*. This figure includes the pharmacological compounds PI-103, TORIN1, GSK650394 and also rapamycin which were used to investigate the role of the PI3K/SGK1 pathway in ENaC expression in both GC-deprived and GC-treated H441 cells. The objectives of this study are listed below:

1) To determine the effect of dexamethasone on P13K and SGK1 activity.
2) To observe the relationship between PI3K, TORC2 and SGK1 activity.
3) To test the hypothesis that P13K/SGK1 activity is vital for the hormonal control of ENaC expression via GC.

4.2 Experimental design

Cells were cultured on 6-well plates and processed as described in *Chapter 3* to determine two different pools of proteins; the endogenous total extract and surface-labelled proteins. PI-103, TORIN1, GSK650394 and rapamycin were used as described in *Chapter 2*. All pharmacological inhibitors were used for approximately 3 h.

In this chapter, the effects of these inhibitors were observed prior to ENaC expression by dexamethasone. All inhibitors (except rapamycin) have been shown to physiologically abolish ENaC activity in dexamethasone-induced cells (Watt, 2011). Therefore a strictly controlled experiment was required to further examine the phosphorylation of the endogenous protein kinases down-stream of the PI3K/SGK1 pathway.

To complement this study, the period of dexamethasone incubation was reduced to 3 h. This will be used as a control for the longer period of incubation (24 h).The expression of each

subunit and how they differ in the presence of compounds that specifically inhibit the PI3-Kinase (PI-103) and SGK1 (GSK650394) pathway will be monitored. This is conducted by assessing the maximal activity of the SGK1 protein indicator, P-NDRG1-Thr$^{346/356/366}$ as seen in *Chapter 3*. Hopefully, this will provide an insight into the response of both dexamethasone and the elected inhibitors. The summary of the experimental design is depicted in *Figure 4-2*.

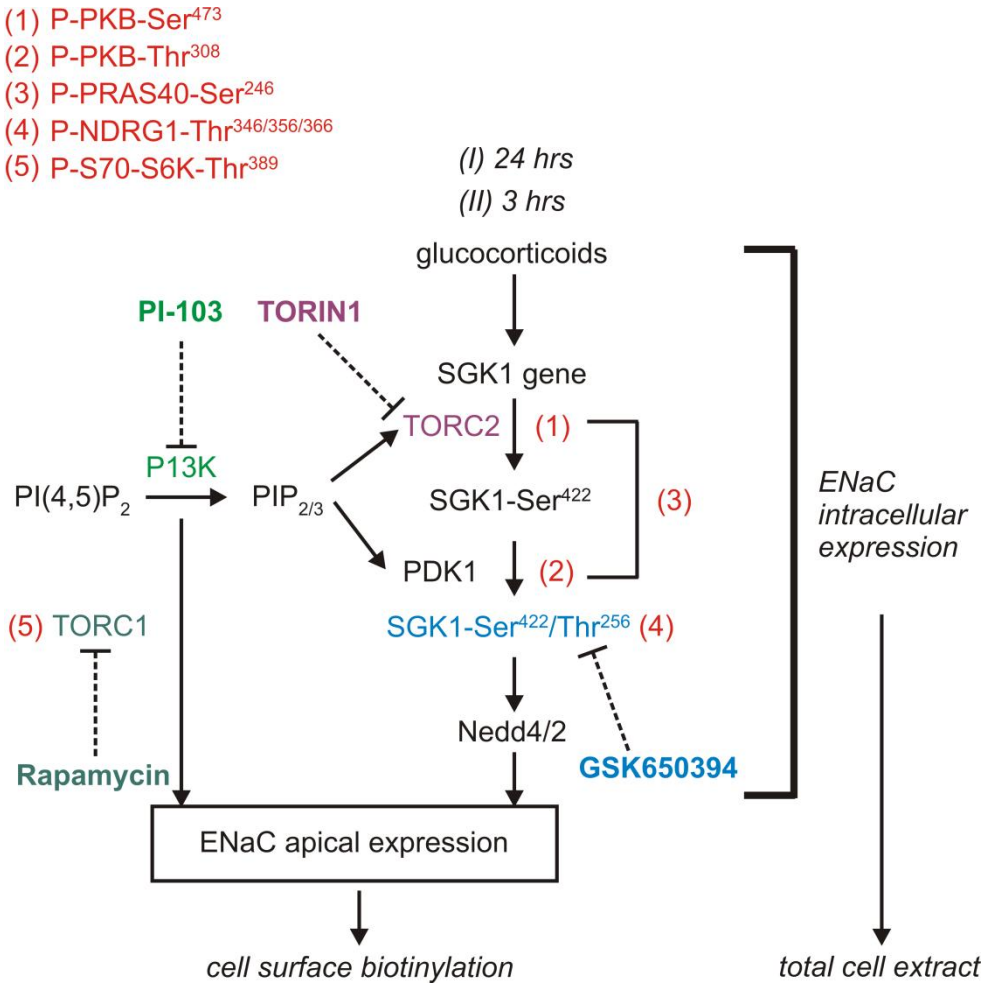

Figure 4-2: The experimental design used to determine the role of the PI3K/SGK1 pathway in ENaC expression.

4.3 Results

4.3.1 Effects of the P13-kinase inhibitor (PI-103) on protein kinases in cells treated with dexamethasone for 24 h

Figure 4-3A proposes that PI-103 selectively inhibits the PI3-kinase and SGK1 pathway (Raynaud *et al.*, 2007; Wang *et al.*, 2008; Garcia-Martinez & Alessi, 2008; McTavish *et al.*, 2009; Mansley & Wilson, 2010a). This substance has been shown to inhibit PI3-kinase and thus deactivate the protein kinases downstream of this pathway that connect to SGK1. ENaC expression is thought to be dependent on this collaborative pathway so induction of this pharmacological component should decrease/abolish the surface expression of ENaC.

Figure 4-3B shows the results of the strictly paired protocol used to study the selective effect of PI-103 when under prolonged incubation with dexamethasone (24 h). Based on the mechanism proposed in *Figure 4-1*, dexamethasone activates the phosphorylation of PKB-Thr^{308} and PKB-Ser^{473} indicating the activation of PDK1 and TORC2, respectively, as found previously in our lab (Mansley & Wilson, 2010b; Watt, 2011). This compound also influences the expression of SGK1, shown by the activation of the Thr$^{346/356/366}$ phosphorylated form of NDRG1. Therefore, these results indicate that dexamethasone affects the PI3K/SGK1 pathway by elevating the expression of the protein kinases that underlie this mechanism.

As this activation by dexamethasone occurs, the presence of PI-103 inhibits TORC2 by abolishing the phosphorylation of PKB-Ser^{473} (Mansley & Wilson, 2010a; Watt, 2011). This also greatly diminishes the expression of PDK1 and SGK1 through the de-phosphorylation of PKB-Thr^{308} and NDRG1-Thr$^{346/356/366}$, respectively. Prior to this, PI-103 had no effect on the total protein.

4.3.2 Effects of the P13-kinase inhibitor (PI-103) on ENaC expression in cells treated with dexamethasone for 24 h

To investigate the correlation between SGK1 pathway activation and ENaC expression at the cell surface, cells were biotinylated and extracted after prolonged exposure to dexamethasone (24 h). As shown in the previous chapter, the two isoforms of α-ENaC showed an increase in expression in both the total lysates and biotinylated surface proteins. Dexamethasone also increased the expression of β-ENaC in the lysates but not at the cell surface, though a single form of γ-ENaC had a significant effect on the surface-labelled protein. Therefore, from this we can deduce that exposing the cells to dexamethasone for a longer period elevates the expression of α-ENaC and γ-ENaC at the cell surface but not β-ENaC. Whilst the specific P13-kinase inhibitor has been shown to inhibit the endogenous protein kinases involved in the PI3K/SGK1 pathway (Bayascas & Alessi, 2005; Mansley & Wilson, 2010a; Watt, 2011), it only has a minor effect on ENaC expression in dexamethasone treated cells (*Figure 4-4B*). As shown previously, two isoforms of α-ENaC are expressed in the presence of dexamethasone, and PI-103 only reduces the full length version (~95 kDa). The expression level was similar for the two different pools: the total lysates and the biotinylated surface protein (*Figure 4-4B*). Treatment with dexamethasone resulted in an elevation in β-ENaC expression in the total lysates but not at the cell surface, but PI-103 had no effect on the surface abundance or the total lysates (*Figure 4-4B*). PI-103 also had no effect on the two isoforms of γ-ENaC in the total lysates but did have a residual effect (yet statistically significant) on the single isoform in the surface-labelled pool of protein (*Figure 4-4B*).

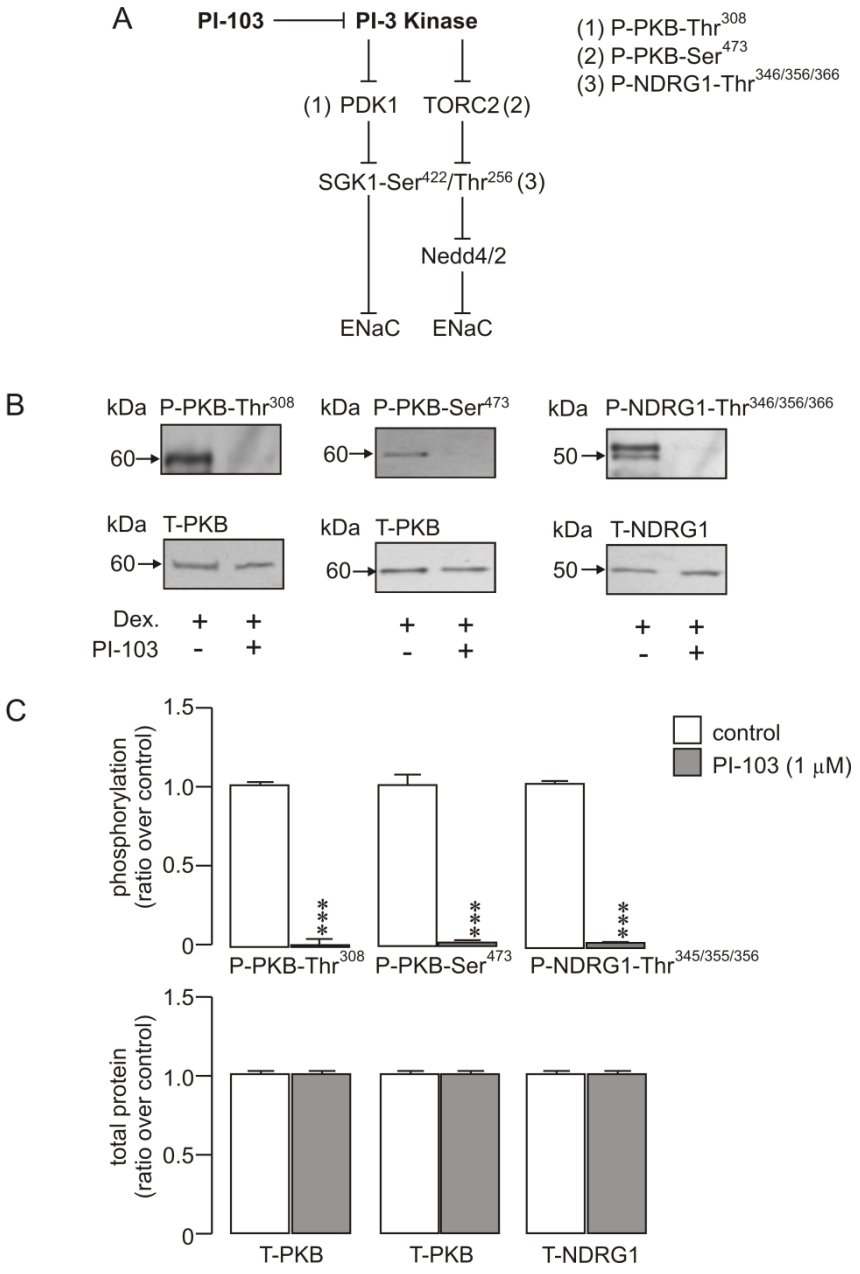

Figure 4-3: Effects of PI-103 on the phosphorylation of PKB-Thr^{308}, PKB-Ser^{473}and NDRG1-Thr$^{346/356/366}$ in dexamethasone treated cells.

(A) A diagram showing the proposed effects of PI-103. (B) Western blots showing the effects of PI-103 (1 μM) on cells induced with dexamethasone (0.2 μM, 24 h) on the overall abundance of protein and the phosphorylation of PKB-Thr^{308}, PKB-Ser^{473} and NDRG1-Thr$^{346/356/366}$, respectively. (C) Bar graphs representing the ratio of the densitometry values of PI-103 over control samples for each of the phosphorylated and total protein kinases. Each experiment was repeated and presented as the mean ± SEM (n = 4). Asterisks denote statistical significance when compared to the control values ***p<0.001 (*Student's* t-test).

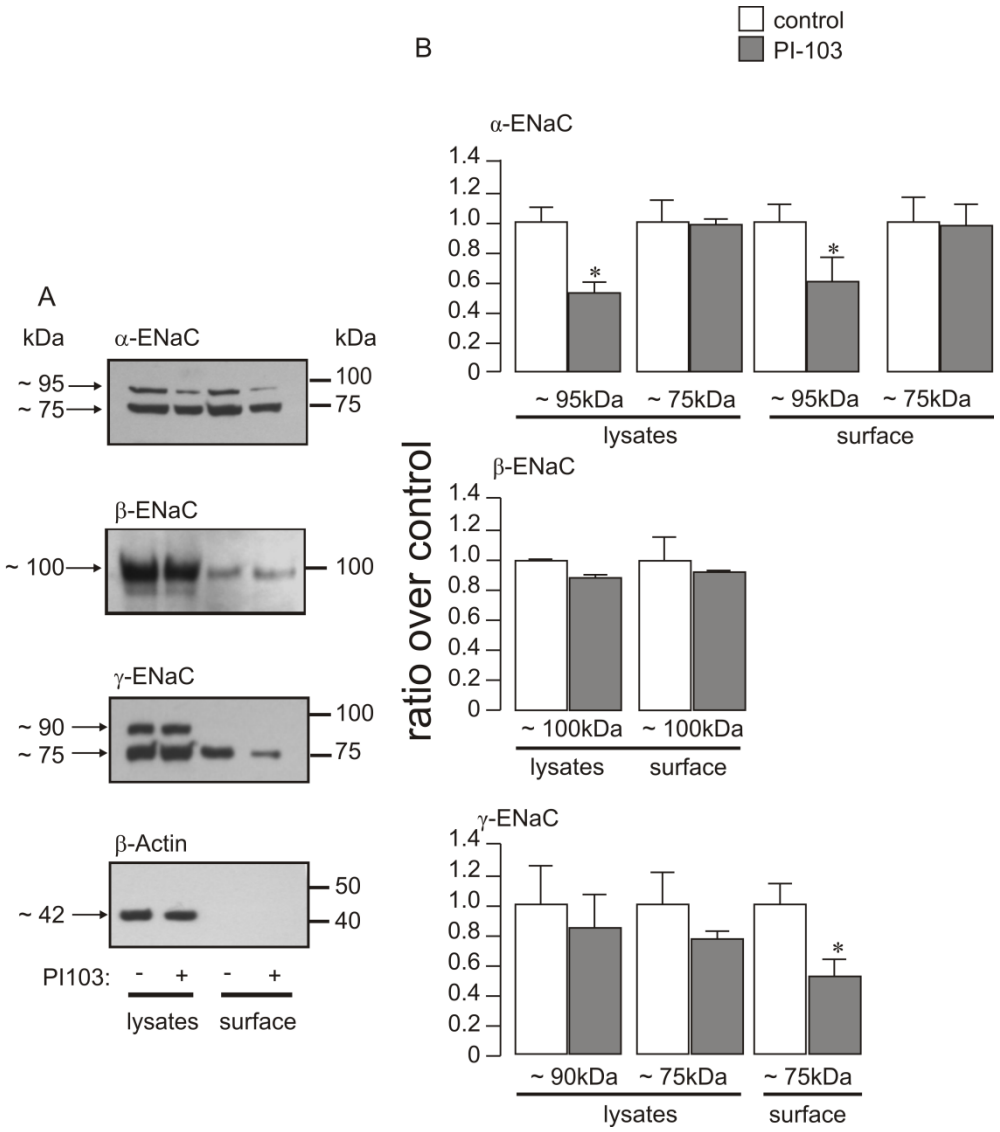

Figure 4-4: Effects of the PI3K inhibitor, PI-103, on ENaC subunits in the presence of dexamethasone (24 h).

(A) Typical Western blots showing the effects of PI-103 (1 μM) on the expression of α-, β-, and γ-ENaC in the presence of dexamethasone (0.2 μM). The cells were maintained in a hormone deprived medium from day 2 until confluent. Dexamethasone was used to induce the cells for 24 h and they were treated with PI-103 for the final 3 h in the presence of DMSO. Actin is shown as a loading control. (B) Densitometric analysis of the data from (A), showing the effects of dexamethasone on the cellular abundance of α-ENaC subunits for ~95 kDa (the upper band) and ~75 kDa (the lower band), the single band (~100 kDa) of β-ENaC, the two isoforms (~90 kDa, ~75 kDa) of γ-ENaC as well as the single isoform from the surface protein. Data are normalised to the control sample and presented as the mean ± SEM (n = 4). The asterisk denotes statistical significance when compared to control cells *p<0.05 (*Student's* t-test).

4.3.3 Effects of the TORC2 inhibitor (TORIN1) on protein kinases in cells treated with dexamethasone for 24 h.

A similar experimental design to the one described previously was applied to the cells treated with dexamethasone (~24 h). TORIN1 (0.1 µM) was added for the final 3 h of the incubation period. This compound is highly potent and selectively inhibits TORC complexes to a far greater extent than rapamycin (Thoreen *et al.*, 2009). PI3-kinase has been shown to have control over the phosphorylation of SGK1 (Kobayashi & Cohen, 1999; Park *et al.*, 1999), and this seems to be mediated by TORC2 (Guertini & Sabatini, 2007; Thoreen *et al.*, 2009) to further down-regulate the SGK1 pathway.

Figure 4-5A depicts the proposed effect of TORIN1 on the pathway. Since this compound has been shown to abolish TORC2 through the protein indicator PKB-Ser^{473} only at a lower concentration (0.1 µM) (Thoreen *et al.*, 2009; Mansley & Wilson, 2010b; Watt, 2011), it may lead to the de-phosphorylation of NDRG1-Thr$^{346/356/366}$ down-stream of the SGK1 pathway, thus decreasing ENaC expression at the cell surface.

As seen in *Figure 4-5B*, TORIN1 abolishes the phosphorylation of PKB-Ser^{473} marking TORC2's inhibition, and this effect significantly reduces the phosphorylation of the SGK1 protein indicator; NDRG1-Thr$^{346/356/366}$. However, the de-activation does not affect the endogenous total protein. Whilst the lower concentration of TORIN1 successfully inhibited the protein indicator of TORC2, it also inhibited the protein indicator for PDK1, a kinase that is activated in a pathway downstream of PI3-kinase. The expression of phosphorylated-PKB-Thr^{308} opposes the present findings from our lab as they have found that TORIN1 has significantly reduced the phosphorylation of this protein kinase (Mansley & Wilson, 2010b; Watt, 2011) and Thoreen *et al.*, (2009). This discrepancy suggests that the selectivity of TORIN1 is broad and that it is somewhat similar to the effects of PI-103. Subsequently, this

drug cannot be used to silence the activation of TORC2-SGK1 and needs to be excluded from further study.

4.3.4 Effects of the TORC2 inhibitor (TORIN1) on ENaC expression in cells treated with dexamethasone for 24 h.

Despite the issues mentioned above, we investigated the effects of TORIN1 on the ENaC subunit, as it was crucial to determine whether TORIN1 had an effect on ENaC similar to that of PI-103. The expression of two isoforms of α-ENaC and a single band of β-ENaC were noted in both the total lysates and the cell surface. Two isoforms of γ-ENaC were expressed in the total cellular extract but only a single band was found from the pool proteins of the cell surface (*Figure 4-6A*). As seen in *Figure 4-6*, TORIN1 reduces the full length isoform of α-ENaC (~95 kDa) in both sets of pool proteins from the H441 cells. It has no effect on β-ENaC (~100 kDa) but results in a smaller band for γ-ENaC (~75 kDa) from the surface pool proteins (*Figure 4-6B*). This data is similar to that showing the effects of PI-103, therefore this compound is not suitable for any future experiments in this study.

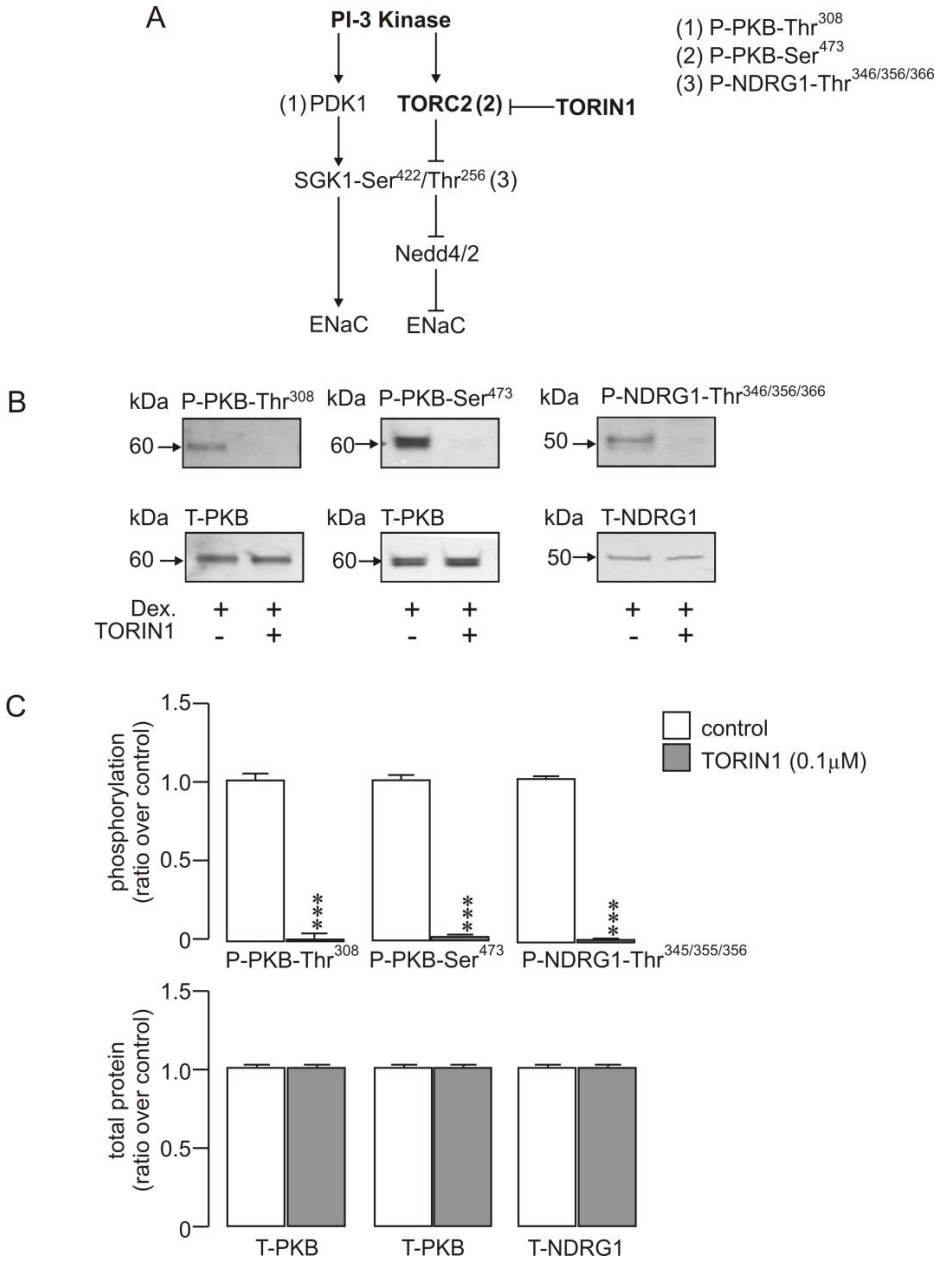

Figure 4-5: Effects of TORIN1 on the phosphorylation of PKB-Thr^{308}, PKB-Ser^{473}and NDRG1-Thr$^{346/356/366}$ in dexamethasone-treated cells.

(A) The proposed effects of TORIN1. (B) Western blots showing the effects of TORIN1 (0.1 μM) on cells induced with dexamethasone (0.2 μM, 24 h) on the overall abundance of protein, and phosphorylation of PKB-Thr^{308}, PKB-Ser^{473} and NDRG1-Thr$^{346/356/366}$, respectively. (C) Bar graphs showing the ratio of densitometry values of TORIN1 over control samples. Each experiment was repeated four times and presented as the mean ± SEM (n = 4). Asterisks denote statistical significance when compared to the initial values, ***p<0.001 (*Student's* t-test).

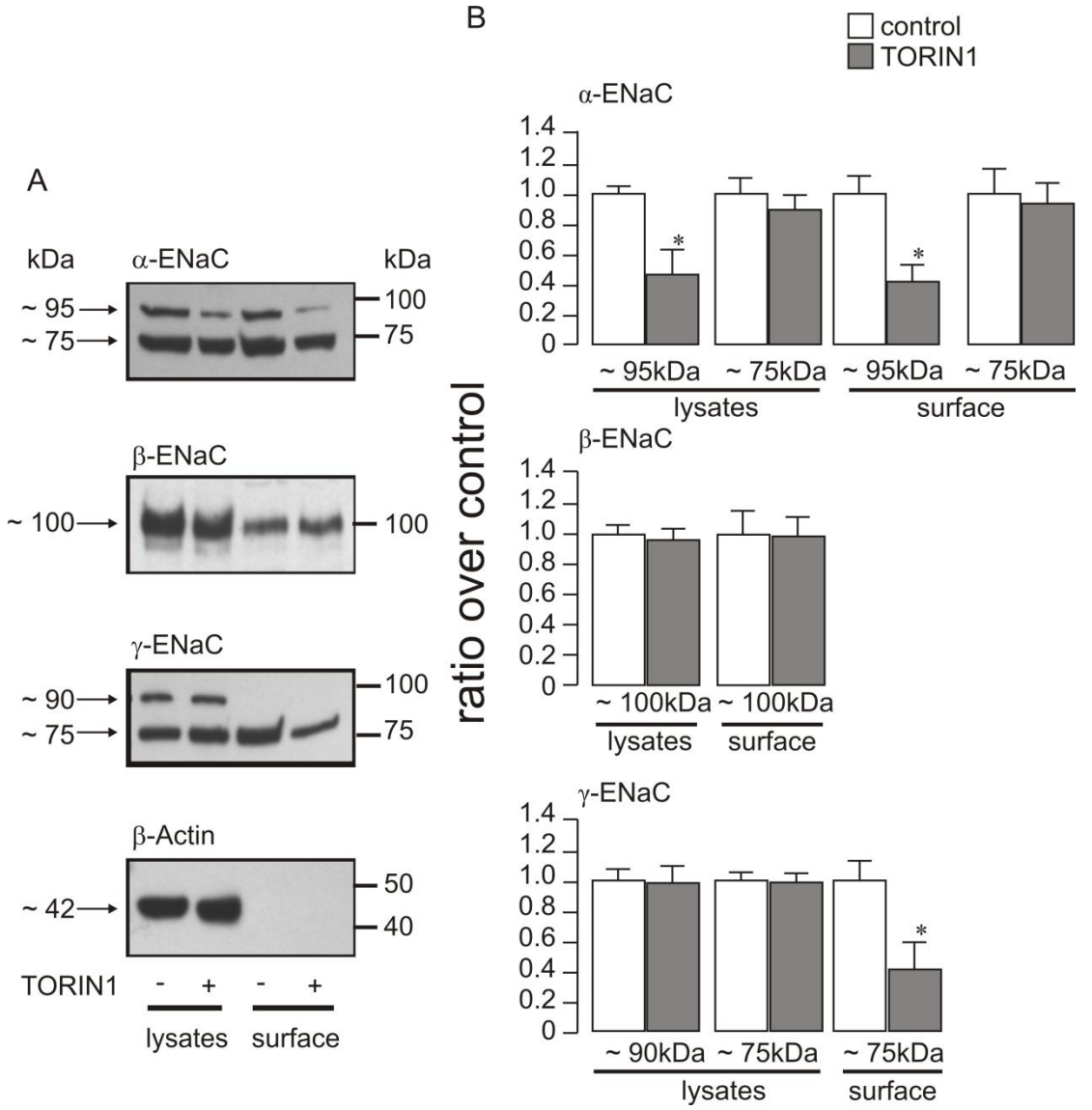

Figure 4-6: Effects of TORIN1 on the expression of ENaC subunits in the presence of dexamethasone (24 h).

(A) Typical Western blots showing the effects of TORIN1 (0.1 µM) on the expression of α-, β-, and γ-ENaC in the presence of dexamethasone (0.2 µM). The cells were maintained in a hormone deprived medium from day 2 until confluent. The cells were incubated with dexamethasone for 24 h, and further treated with TORIN1 for 3 h in the presence of DMSO. Actin is shown as a loading control. (B) Densitometric analysis of data showing the effects of dexamethasone on the cellular abundance of ENaC subunits. Data are normalised to the control and presented as the mean ± SEM (n = 4). Asterisks denote statistical significance when compared to the initial values ***p< 0.001 (*Student's* t-test).

4.3.5 Effects of the SGK1 inhibitor (GSK650394) on protein kinases in cells treated with dexamethasone for 24 h

The pharmacological substance GSK650394 is a recently developed drug that has been shown to successfully abolish SGK1 activity (Sherk *et al.*, 2008; Lang *et al.*, 2009; Lang & Gorlach, 2010; Mansley & Wilson, 2010a; Watt, 2011). *Figure 4-7A* shows the proposed effects of this compound, which is thought to act by inhibiting the phosphorylation of NDRG1-Thr$^{346/356/366}$ in the SGK1 pathway, thereby indicating the de-activation of SGK1. As a consequence of this, ENaC expression would decrease and be absent from the cell surface.

Figure 4-7B shows the effects of GSK650394 (10 µM) and reveals no effect on PKB-Thr^{308}, indicating that PDK1 is still active when the SGK1 is not active. Additionally, there was no change in the phosphorylation of PRAS40-Ser^{246}, which is an indicator for the activation of PKB (Fonseca *et al.*, 2007; Miller *et al.*, 2008; Mansley & Wilson, 2010a; Nascimento *et al.*, 2010; Watt, 2011). Therefore, this compound specifically inhibits the phosphorylation of NDRG1-Thr$^{346/356/366}$; a protein that indicates SGK1 activity, as shown previously by our lab (Mansley & Wilson, 2010a; Watt *et al.* 2012). Although this inhibition is specific, it also appears to de-phosphorylate the Ser^{473} residue of PKB. This is unexpected, however such effect has also been found previously in a mouse kidney cell line (Mansley & Wilson, 2010a). Apart from this, no change in the total protein was observed, confirming that this compound reliably and specifically reduces/abolishes the phosphorylation of PKB.

4.3.6 Effects of the SGK1 inhibitor (GSK650394) on ENaC expression in cells treated with dexamethasone for 24 h.

To further investigate the effects of GSK650394, we examined ENaC expression in H441 cells, *Figure 4-8* illustrates the expression of the ENaC subunits in the presence of this inhibitor. Dexamethasone is known to increase levels of α-ENaC and γ-ENaC, but not the β- subunit, at the cell surface. As seen previously with both PI-103 and TORIN1, GSK650394 reduces the full length of α-ENaC (~95 kDa) in both pools of proteins, whilst showing a slight decrease in the single isoform of γ-ENaC (~75 kDa). However, such effects were not detectable for β-ENaC, in either the total lysate or the surface pool of proteins. Whilst GSK650394 is known to reduce ENaC activity in dexamethasone-induced H441 cells (Watt, 2011), the data shown here only reveals a minor effect on overall ENaC expression. Therefore, we cannot explain the relationship between ENaC expression and its activity through amiloride sensitive Na^+ current.

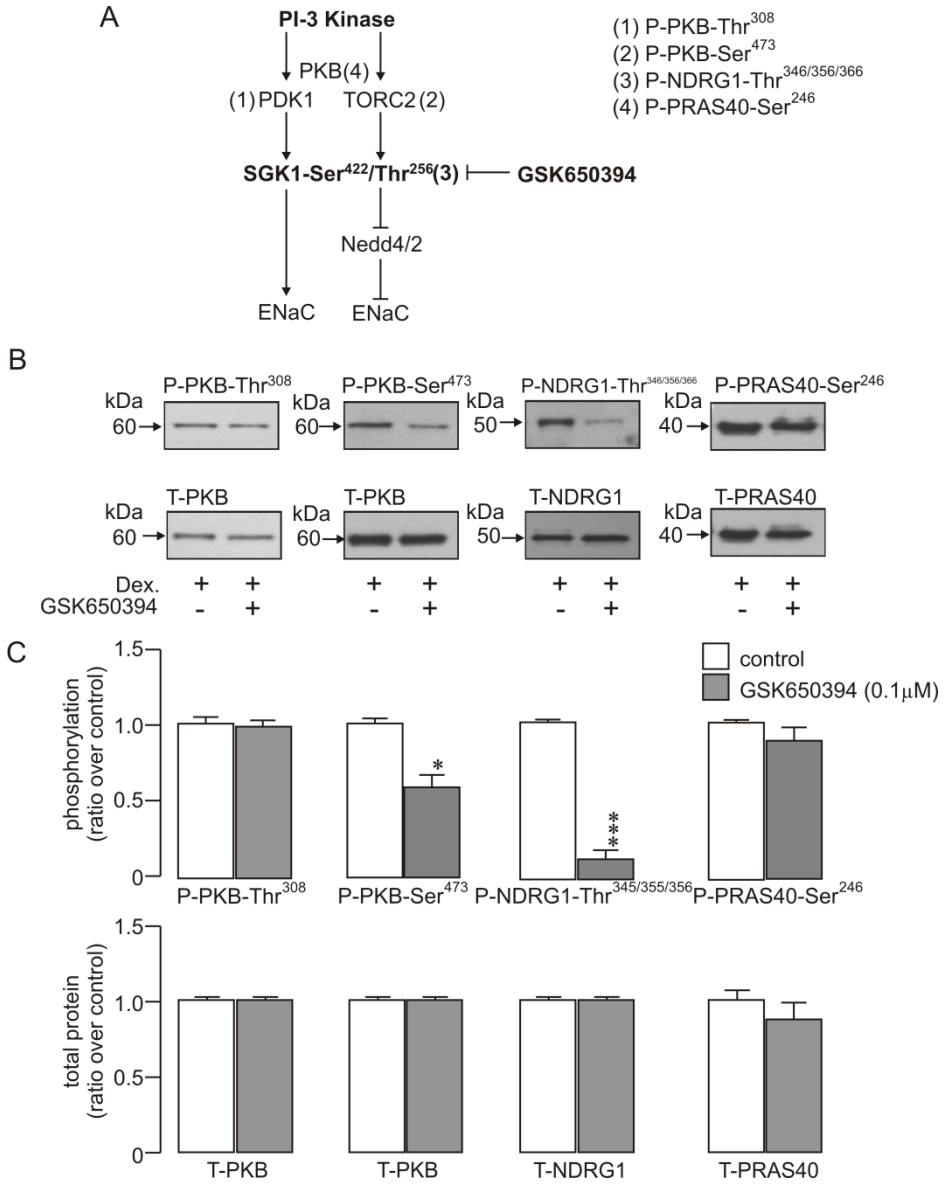

Figure 4-7: Effects of GSK650394 on the phosphorylation of PKB-Thr^{308}, PKB-Ser^{473}, NDRG1-Thr$^{346/356/366}$ and PRAS40-Ser^{246} in dexamethasone-treated cells.

(A) The proposed effects of GSK650394. (B) Western blots showing the effects of GSK650394 (0.1 μM) in dexamethasone-treated cells (0.2 μM, 24 h) on the overall abundance of protein and the phosphorylation of PKB-Thr^{308}, PKB-Ser^{473}, NDRG1-Thr$^{346/356/366}$ and PRAS40-Ser^{246}, respectively. The cells were induced with dexamethasone and incubated for 24 h, and treated with GSK650394 in the final 3 h. (C) The densitometry values for GSK650394 were calculated as the ratio over the control sample. The results are shown as bar graphs for both the phosphorylated and the total protein kinases. Each experiment was repeated four times and presented as the mean ± SEM (n = 4). Asterisks denote statistical significance when compared to control samples; *p<0.05, ***p<0.001 (*Student's* t-test).

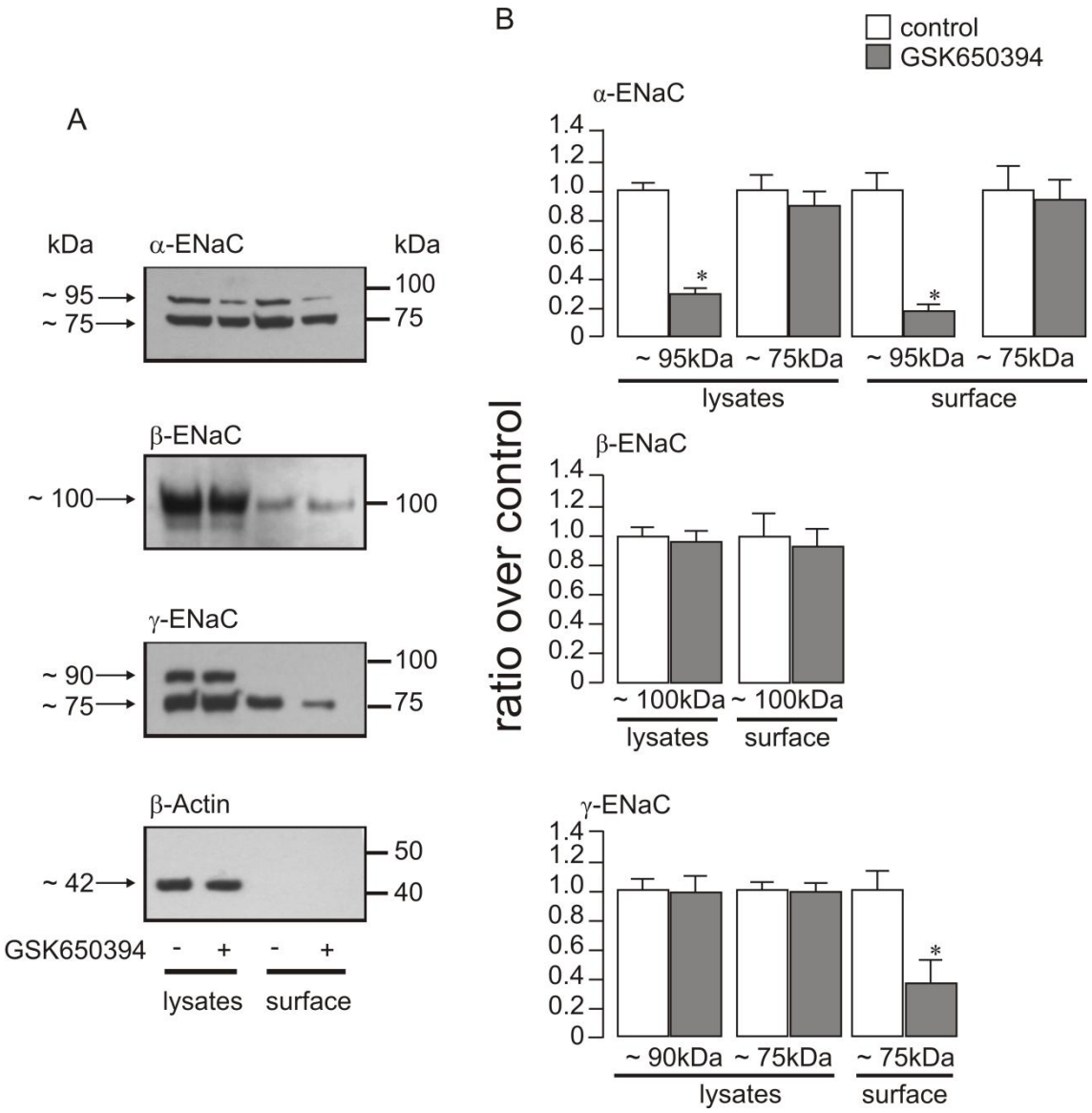

Figure 4-8: Effects of the SGK1 inhibitor, GSK650394, on the expression of ENaC subunits in the presence of dexamethasone (24 h).

(A) Typical Western blots showing the effects of GSK650394 (10 µM) on the expression of α-, β-, and γ-ENaC in the presence of dexamethasone (0.2 µM). The cells were maintained in a hormone deprived medium from day 2 until confluent. The cells were induced with dexamethasone and incubated for 24 h and treated with GSK650394 for another 3 h. Actin is shown as a loading control. (B) Densitometric analysis of the data in (A) showing the effects of dexamethasone on the cellular abundance of ENaC subunits. Data are normalised to the control and presented as the mean ± SEM (n = 4). Asterisks denote statistical significance when compared to the initial values; *p< 0.05, ***p<0.001 (*Student's* t-test).

4.3.7 Effects of the TORC1 inhibitor (rapamycin) on protein kinases in cells treated with dexamethasone for 24 h

Rapamycin does not alter the phosphorylation of PKB-Thr^{308}, PKB-Ser^{473} or NDRG1-Thr$^{346/356/366}$ in GC-stimulated cells (*Figure 4-9*). Since rapamycin is a selective inhibitor for TORC1 (Bain *et al.*, 2007; Proud, 2007; Mansley & Wilson, 2010b), it essentially abolishes the expression of P70-S6K-Thr^{389} with no effect on the overall expression. This demonstrates an exquisite selective control for the inhibition of TORC1, but not the protein kinases related to the SGK1 pathway. Using rapamycin in this experiment reveals the importance of the PI3K/SGK1 pathway in the control of ENaC expression.

4.3.8 Effects of the TORC1 inhibitor (rapamycin) on ENaC expression in cells treated with dexamethasone for 24 h

As rapamycin only inhibits TORC1 and no other kinases, we expected that it would not affect ENaC expression in either pool of proteins. This compound increases cellular efficiency and is widely used as an immunosuppressant. However, treatment with rapamycin caused a reduction in the full length of α-ENaC (*Figure 4-10*), but not β- and γ-ENaC. This was unanticipated and the effects were similar to that seen with PI-103, TORIN1, and GSK650394, even though rapamycin does not have an effect on ENaC activity (Watt, 2011). Therefore, this data suggests that TORC1 may have an effect on ENaC, especially α-ENaC. However, the mechanism that underlies this process still requires clarification.

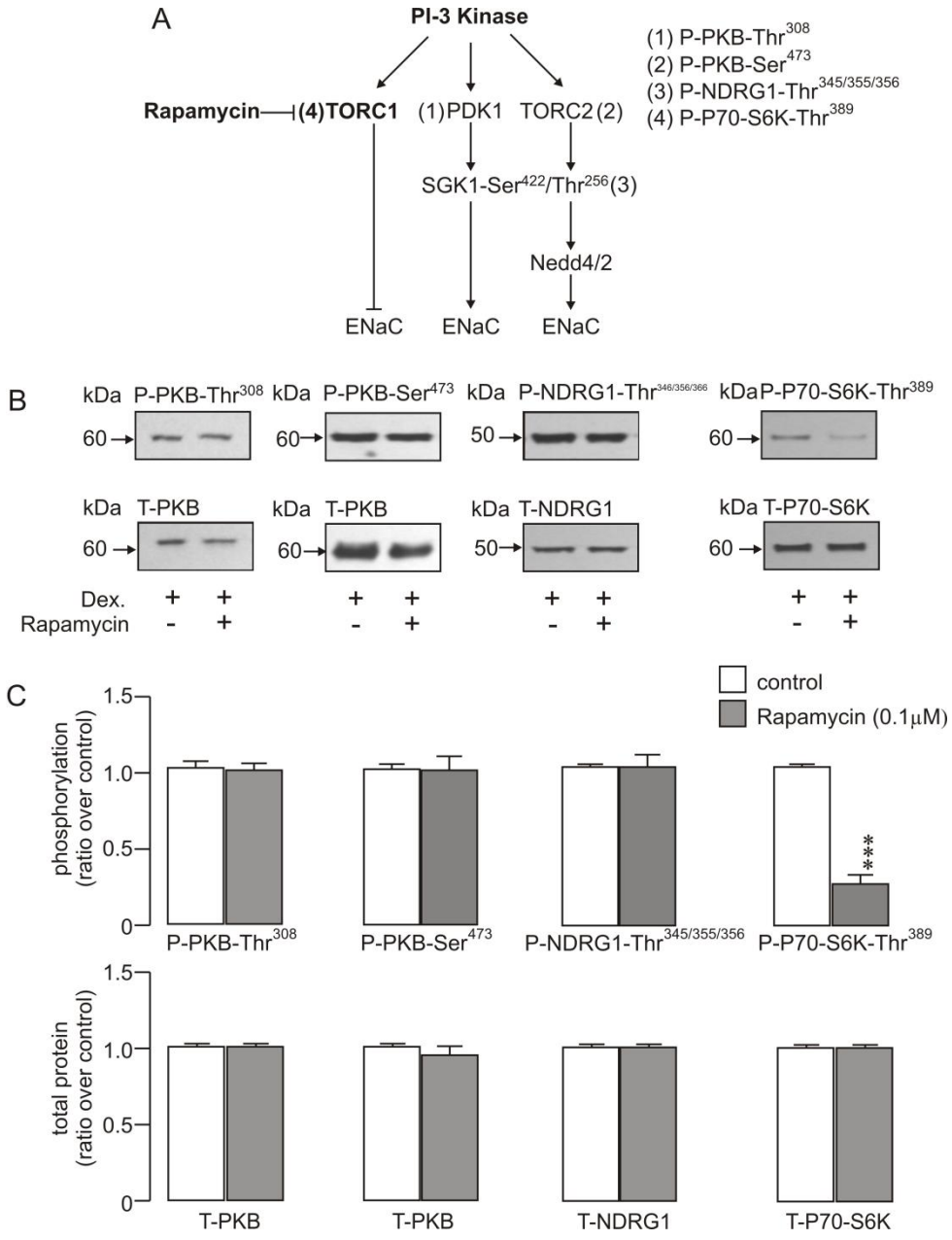

Figure 4-9: Effects of rapamycin on the expression of PKB-Thr^{308}, PKB-Ser^{473} and NDRG1-Thr$^{346/356/366}$ phosphorylation in dexamethasone-treated cells.

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(A) The proposed effects of rapamycin. (B) Western blots showing the effects of rapamycin (0.1 μM) in cells induced by dexamethasone (0.2 μM, 24 h) on the overall abundance of protein and phosphorylation of PKB-Thr^{308}, PKB-Ser^{473}, NDRG1-Thr$^{346/356/366}$ and P70-S6K-Thr^{389} , respectively. (C) The bar graphs depict the densitometry values of rapamycin treatment calculated as a ratio over the control samples. Each experiment was repeated four times and presented as the mean ± SEM (n = 4). Asterisks denote statistical significance when compared to control values, ***p<0.001 (*Student's* t-test).

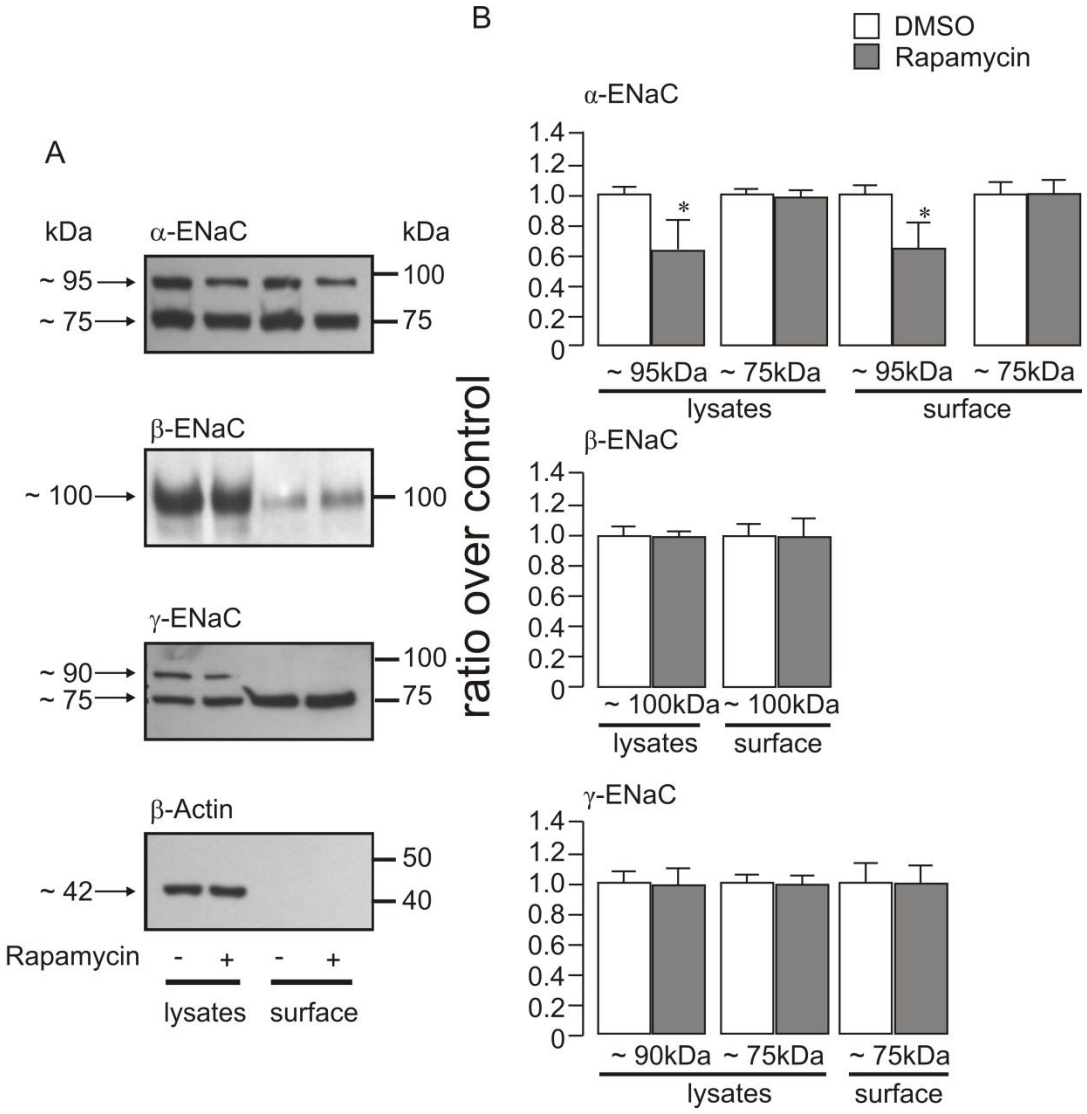


Figure 4-10: Effects of the TORC1 inhibitor, rapamycin, on the expression of ENaC subunits in the presence of dexamethasone (24 h).

(A) Typical Western blots showing the effects of rapamycin (0.1 µM) on the expression of α-, β-, and γ-ENaC in the presence of dexamethasone (0.2 µM). The cells were maintained in a hormone deprived medium from day 2 until confluent. The cells were induced with dexamethasone and incubated for 24 h, and treated with rapamycin for the final 3 h in the presence of DMSO. Actin is shown as a loading control. (B) Densitometric analysis of the data in (A) showing the effects of dexamethasone on the cellular abundance of ENaC subunits. Data are normalised to the control and presented as the mean ± SEM (n = 4). Asterisks denote statistical significance when compared to the initial values ***p<0.001 (*Student's* t-test).

4.3.9 Dexamethasone transiently activates ENaC

Dexamethasone elevated SGK1 activity and this can be indicated by a transient increased phosphorylation of NDRG1-Thr$^{346/356/366}$ up to the 3 h point, then SGK1 activity decreased for the remaining incubation time (up to 24 h; *Figure 4-13A*), confirming that found by Inglis *et al.*, (2009) and Watt, (2011). The GC-induced cells activated SGK1 and activity peaked after a brief exposure (3 h) to dexamethasone. This activation also evoked expression of the ENaC subunits (α-, β-, γ-) at the cell surface. The level of each protein was significantly increased by dexamethasone, returning to the basal level after 24 h with the exception of α-ENaC and γ-ENaC which persisted when exposed to prolonged treatment (*Figure 4-11B*).

The augmentation of ENaC indicates that brief exposure to dexamethasone could increase SGK1 activity, and thus elevate ENaC expression towards the membrane. However, the transient effect of dexamethasone cannot induce ENaC activity in H441 cells (Watt, 2011). This suggests that a brief activation of SGK1 mediates the trafficking of each subunit towards cell surface, but that over time the functional effect of ENaC can only be seen with both α- and γ-ENaC. The increase in α-ENaC occurs in parallel to the elevation of α-ENaC mRNA (Nakamura *et al.*, 2002; Mustafa *et al.*, 2004) and the α-ENaC gene promoter (McTavish *et al.*, 2009). However, the increment of γ-ENaC is only residual, this reflects the modulation of its encoded mRNA through the influence of GCs (Itani *et al.*, 2002b).

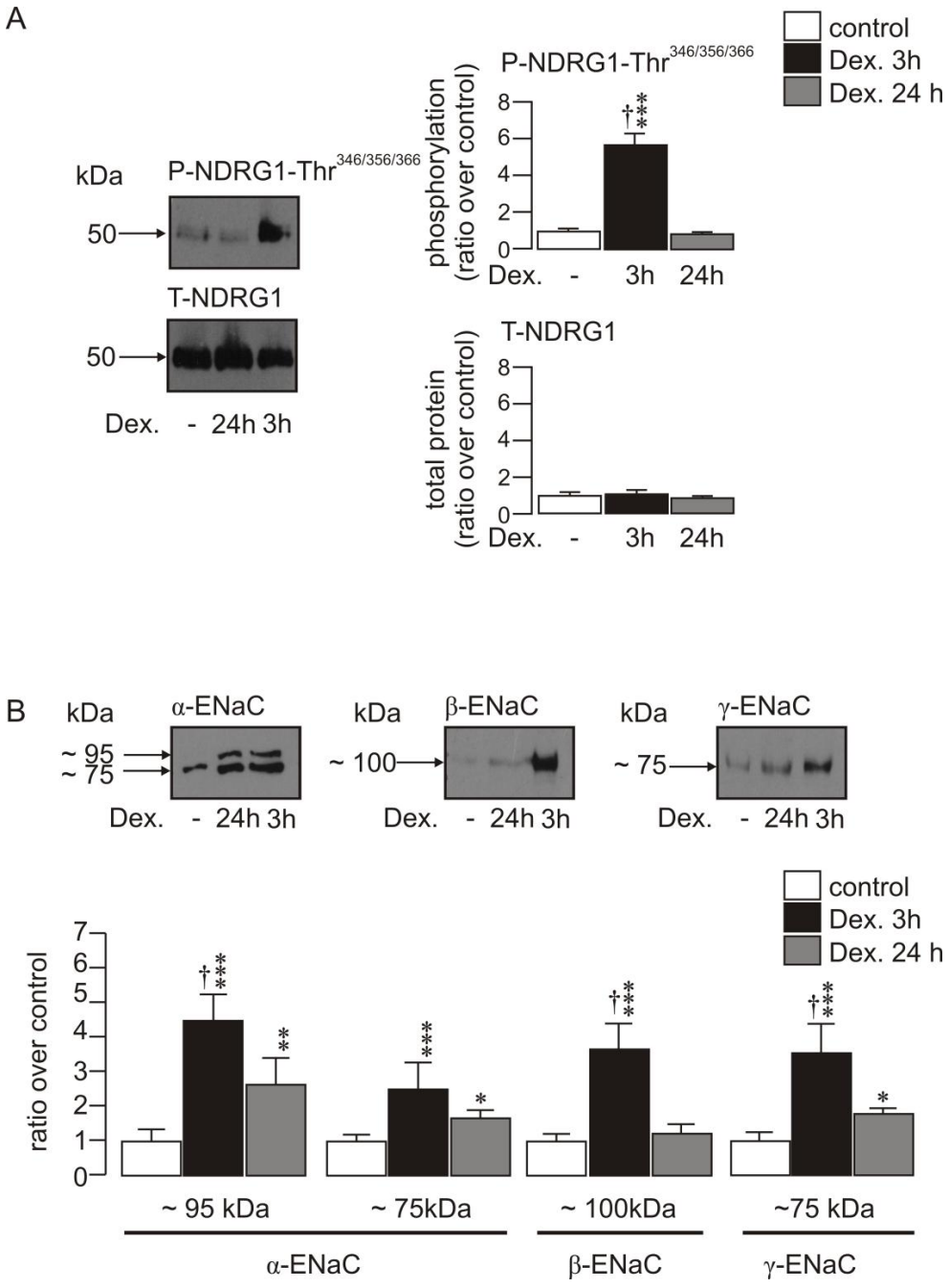


Figure 4-11: The expression of the α-, β- and γ- subunits of ENaC at two different time-points; 24 h and 3 h in dexamethasone-treated H441 cells.

(A) Typical Western blotting using cell surface biotinylation of dexamethasone-treated H441 cells that have undergone brief (3 h) and chronic (24 h) treatment. These blots are representative of three independent samples. (B) Densitometric analyses of the Western blotting in (A). *p<0.05, **p<0.01 and ***p<0.001 are statistically different to GC-deprived cells. † indicates significant difference between chronic (24 h) and brief treatments (3 h); †p<0.05. (one-way ANOVA, Bonferroni *post-hoc* test).

4.3.10 Effects of PI-103 and GSK650394 on ENaC expression after cells are briefly exposed to dexamethasone

Subsequent results revealed two important compounds (PI-103, *Figure 4-12;* and GSK650394, *Figure 4-13*) that are known to knock-out PI3-kinase and SGK, respectively. Brief treatment with dexamethasone (3 h) resulted in an increase in ENaC cell surface expression. By using these compounds to block the PI3-kinase and SGK1 pathways the transient effect of dexamethasone on ENaC expression at the cell surface was revealed.

PI-103 successfully reduces the expression of each subunit of ENaC at the cell surface when briefly exposed to dexamethasone. However, in GC-deprived cells, this compound seems to decrease the expression of both α-ENaC isoforms, though there is no obvious effect on β- and γ-ENaC *(Figure 4-12)*.

The SGK1 inhibitor, GSK650394 (10 μM) reduces the two isoforms of α-ENaC (*Figure 4-13)* but completely abolishes β- and γ-ENaC in cells when treated with dexamethasone for 3h. In GC-deprived cells, the substance reduces the expression of the full length α-ENaC and β-ENaC but not γ-ENaC.

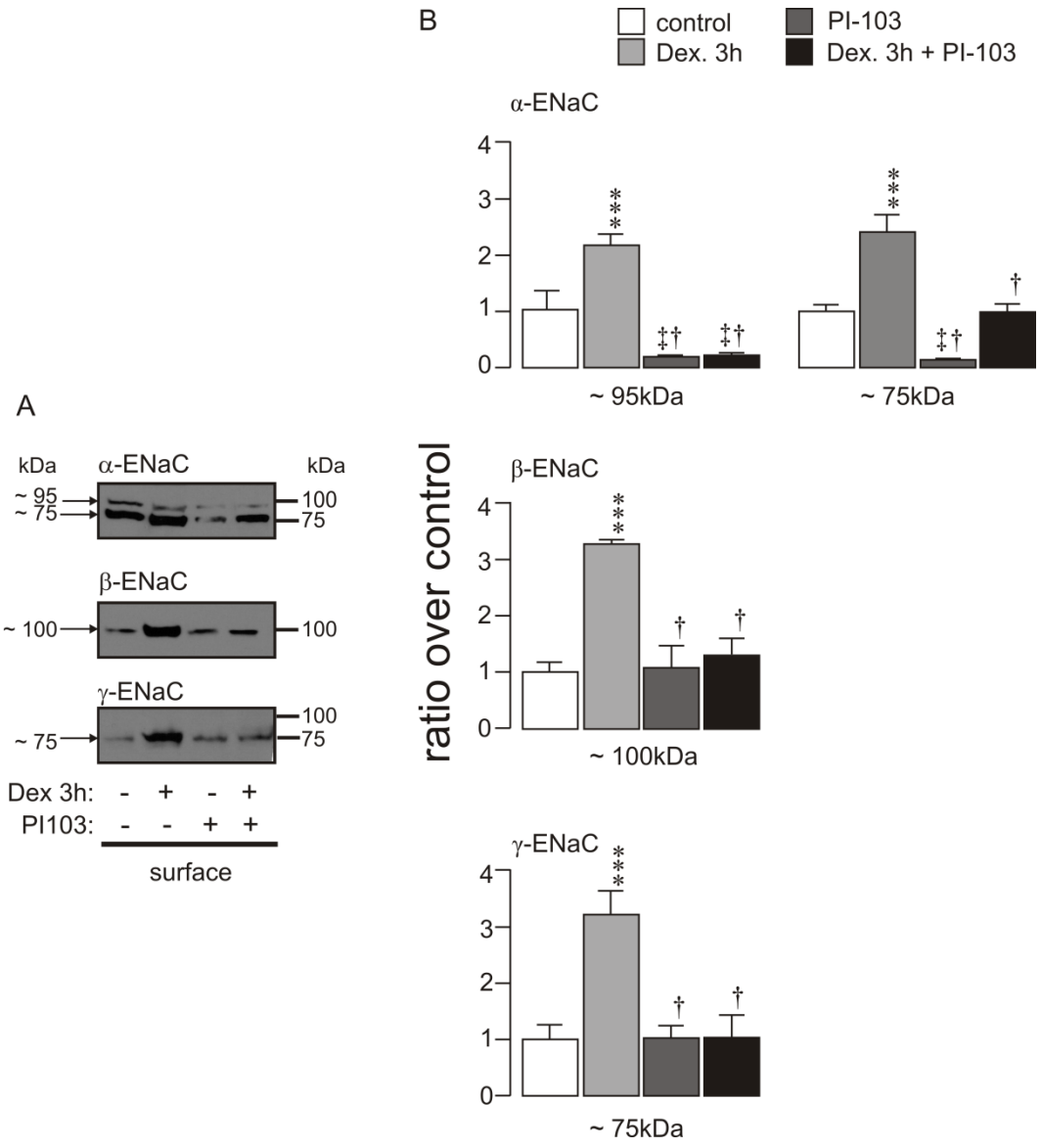


Figure 4-12: Effects of the PI3K inhibitor, PI-103 on ENaC subunits in the presence of dexamethasone (3 h).

(A) Typical Western blots showing the effects of PI-103 (1 μM) on expression of α-, β-, and γ-ENaC in the presence of dexamethasone (0.2 μM). Dexamethasone and PI-103 were brought together for the final 3 h of incubation in the presence of DMSO. Cell surface proteins were extracted and subjected to Western analysis (B) Densitometric analysis of the data in (A) showing the effects of PI-103 on the cellular abundance of α-ENaC subunits: ~95 kDa (the upper band) and ~75 kDa (the lower band), a single band (~100 kDa) for β-ENaC, two isoforms (~90 kDa, ~75 kDa) for γ-ENaC as well as a single isoform from the surface protein. Data are normalised to the control and presented as the mean ± SEM (n = 4). Asterisks denote statistical significance when compared to control cells, *p<0.05, ***p<0.001; whilst the daggers show the effect of PI-103 in the presence of GC, †p<0.001 and control cells, (‡p<0.001) (one-way ANOVA, Bonferroni *post-hoc*-test).

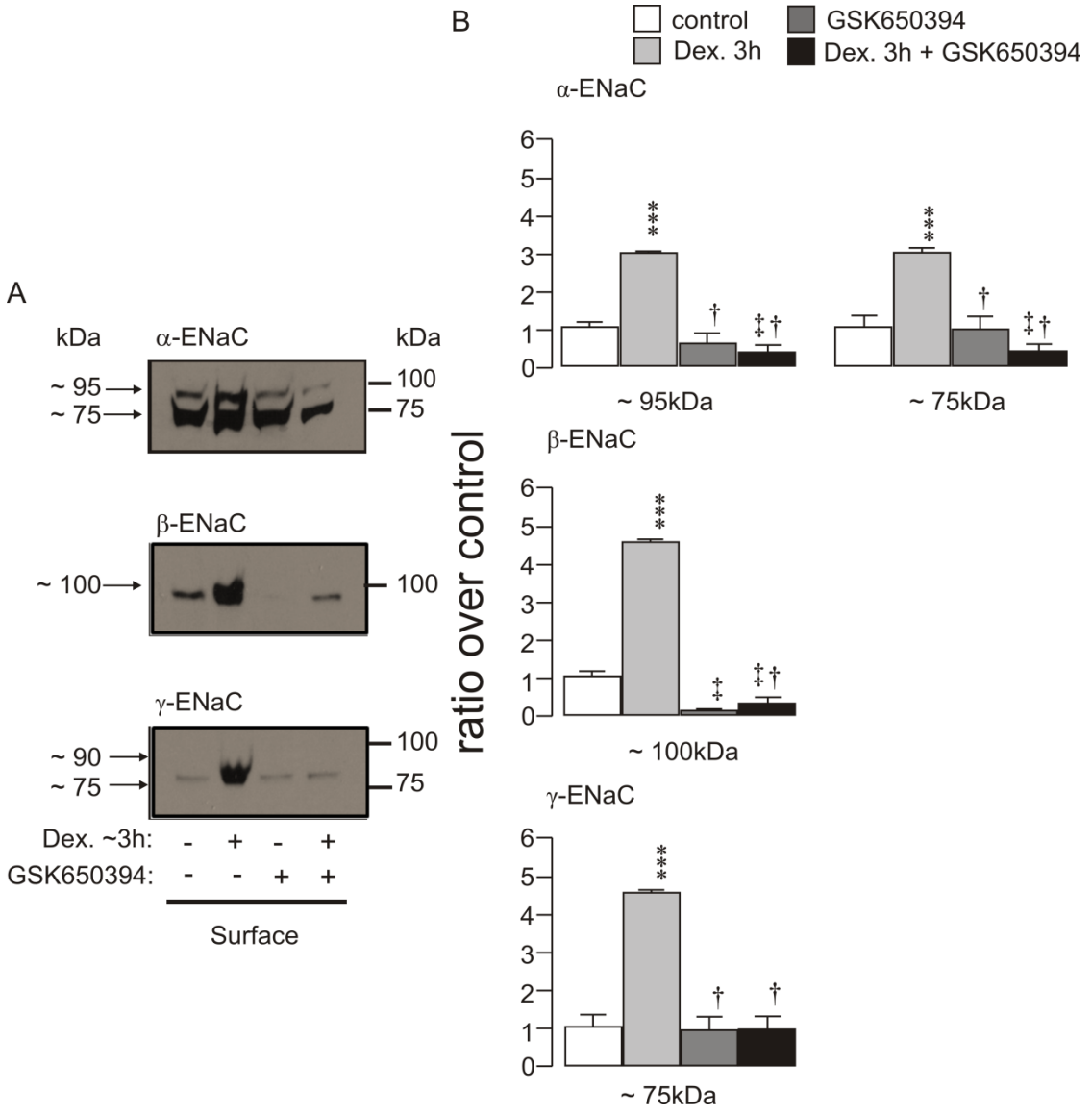


Figure 4-13: Effects of the SGK1 inhibitor, GSK650394, on the expression of ENaC subunits in the presence of dexamethasone (3 h).

(A) Typical Western blots showing the effects of GSK650394 (10 µM) on the expression of α-, β-, and γ-ENaC in the presence of dexamethasone (0.2 µM). The cells were maintained in a hormone deprived medium from day 2 until confluent. Dialysed media was changed 24 h prior to the experiment. Dexamethasone and GSK650394 were used to induce the cells in the final 3 h of incubation in the presence of DMSO. Cell surface proteins were extracted and subjected to Western analyses. (B) Densitometric analysis of the data in (A) showing the effects of GSK650394 on the cellular abundance of α-, β, and γ- ENaC in the surface pool proteins. Data are normalised to the control and presented as the mean ± SEM (n = 4). Asterisks denote statistical significance when compared to control cells; ***p<0.001 whilst the daggers indicate a decreased effect of GSK650394 to the stimulation of dexamethasone (†p<0.001) and control cells (‡p<0.05) (one-way ANOVA, Bonferroni *post-hoc*-test).

4.4 Discussion

Dexamethasone-treated H441 cells activate endogenous ENaC expression that is associated with an increase in membrane Na^+ conductance. The results reported in this chapter confirm that ENaC expression is strictly dependent on GC stimulation (see also Lazrak & Matalon, 2003; Clunes *et al.*, 2004; Brown *et al.*, 2008; Althaus *et al.*, 2010). GCs are physiologically important regulators of pulmonary Na^+ transport and, since these hormones stimulate the removal of fluid from the alveolar space (Matalon & O'Brodovich, 1999; Matthay *et al.*, 2002; Olver *et al.*, 2004), synthetic GCs are used in the clinical management of conditions such as neonatal respiratory distress syndrome and pulmonary oedema that are characterised by the accumulation of liquid in this region of the lung. Moreover, since GCs also exert powerful anti-inflammatory actions, GC receptor agonists are also used in the treatment of lung diseases such as asthma and chronic obstructive pulmonary disease that are characterised by chronic inflammation (Barnes, 2011). It is therefore important to understand the mechanisms that allow pulmonary epithelial cells to respond to these hormones and, in order to study the mechanisms that underlie the activation of endogenous ENaC, we have now explored the effects of small molecule inhibitors of various kinases upon the dexamethasone-dependent Na^+ currents seen in these cells (Watt, 2011). This approach is, however, complicated by the fact that such compounds almost invariably act upon multiple targets (Bain *et al.*, 2007). To control for this, we also used phospho-specific antibodies to monitor the phosphorylation status of endogenous kinase substrates in order to ensure that the tested compounds caused full inhibition of their targets without inactivating other, closely related kinases.

4.4.1 Inhibition of the PI3-Kinase / SGK1 pathway in GC-induced cells by endogenous protein kinases

Pooled data from the endogenous protein extracted from dexamethasone-treated H441 cells revealed the phosphorylation of PKB-Ser^{473}/Thr^{308}, NDRG1-Thr$^{346/356/366}$, PRAS40-Ser^{246} and P70-S6K-Thr^{389}. This indicates that the activation of the P13-kinase/SGK1 pathway occurred as proposed in *Figure 4-1,* and thus confirms the physiological effects of dexamethasone on PDK1 and TORC2 (Watt, 2011). Since these kinases depend on the presence of PIP_2/PIP_3 at the plasma membrane (Biondi *et al.*, 2001; Bayascas & Alessi, 2005; Garcia-Martinez & Alessi, 2008), PI3-kinase seems to be activated under these conditions. These studies revealed activity of SGK1 and PKB; this was anticipated since these regulatory kinases lie downstream of PI3K – TORC2 / PDK1 (Kobayashi & Cohen, 1999; Park *et al.*, 1999; Biondi *et al.*, 2001; Bayascas & Alessi, 2005; Sarbassov *et al.*, 2005; García-Martínez & Alessi, 2008). PI-103 successfully inactivated these substrates which are important downstream targets of PI3-kinase (Kobayashi & Cohen, 1999; Park *et al.*, 1999; Bayascas & Alessi, 2005), including PKB-Ser^{473}/Thr^{308}, NDRG1-Thr$^{346/356/366}$, and did not affect overall protein expression, which happened previously with kidney epithelial cells (Mansley & Wilson, 2010a). This suggests that the compound specifically suppresses the phosphorylation of PDK1 and also inactivates protein kinases downstream of TORC2-SGK1 (see *Figure 4-1*). The inactivation is consistent with the depletion of PIP_2/PIP_3 which leads to decreased activity of PDK1 and TORC2, and thus inactivates SGK1 and PKB (Kobayashi & Cohen, 1999; Park *et al.*, 1999; Bayascas & Alessi, 2005; Sarbassov *et al.*, 2005, Garcia-Martinez & Alessi, 2008).

Our subsequent experiments explored the effects of TORIN1, that inhibits TORC2, the protein that allows PI3K to control both PKB and SGK1 (Sarbassov *et al.*, 2005; García-Martínez & Alessi, 2008; Thoreen *et al.*, 2009). TORIN1 virtually abolished the

phosphorylation of PKB-Ser^{473} as well as Thr^{308} and NDRG1-Thr$^{346/356/366}$ without any effect on the total protein. However, this experiment was performed in a low concentration (0.1 µM) of the substance, since TORIN1 abolishes TORC1/2 complexes and may inhibit PI3-kinase and/or PDK1 at > 1 µM (Thoreen *et al.*, 2009). The data is quite surprising when compared to the findings of Mansley & Wilson (2010b) and Watt (2011), where inhibition was shown to be complete at < 0.1 µM, indicating that PDK1 is still sensitive to this compound at this concentration. One possible explanation is that TORC2 acts as a protein mediator, allowing SGK1 activation through PKB. The mechanism can be explained thus: TORC2 catalyses the phosphorylation of PKB-Ser^{473} to facilitate subsequent activation of PKB-Thr^{308} by PDK1 (Biondi *et al.*, 2001; Sarbassov *et al.*, 2005), so the inhibition of TORC2 may also restrict the phosphorylation of PKB-Thr^{308} (Biondi *et al.*, 2001; Sarbassov *et al.*, 2005). Therefore, this inhibition confirms that TORC2 plays a critical part in the mechanism that allows PI3-kinase to regulate ENaC through the SGK1 pathway (Kobayashi & Cohen, 1999; Park *et al.*, 1999; Garcia-Martinez & Alessi, 2008; Lu *et al.*, 2010). Though TORIN1 does not inhibit PI3-kinase (Thoreen *et al.*, 2009), it may allow the presence of PIP_2/PIP_3 phospholipid second messengers to activate ENaC at the cell surface by binding to the channel complex (Blazer-Yost *et al.*, 2004; Blazer-Yost & Nofziger, 2005; Ma *et al.*, 2007; Pochynyuk *et al.*, 2007a). Where TORIN1 only contributes a minor effect towards ENaC surface expression as seen in the treatment of PI-103, it is implying that PIP_2/PIP_3 still can support ENaC expression when TORC2 is inactive. However, this mechanism must be of secondary importance in H441 cells.

Prior to determining the role of SGK1 in GC-induced ENaC expression, we used a selective inhibitor of SGK1, GSK650394 (Sherk *et al.*, 2008; Mansley & Wilson, 2010a), which has been shown to completely abolish SGK1 protein expression through NDRG1-Thr$^{346/356/366}$ at a concentration of 10 µM (Mansley & Wilson, 2010a). This effect withdraws the amiloride

sensitive Na^+ current (Watt, 2011) suggesting that SGK1 is critical for maintaining ENaC at the cell surface. Apart from this, the compound had a slight effect on PKB-Ser^{473}, may be caused by an inhibitory feedback loop effect via PKB and several AGC kinases between TORC2/SGK1 (Chan *et al.,* 1999). PKB may be involved in the hormonal control of Na^+ absorption in *Xenopus* oocytes and Fischer rat thyroid cells, and it has been suggested that PKB might be involved in the hormonal control of Na^+ transport via ENaC (Lee *et al.,* 2007; Diakov *et al.,* 2010). However, judging by the effect of GSK650394 on PRAS40-Thr^{256} phosphorylation, which indicates PKB activation, this compound had a negligible effect on PKB activity, suggesting that PKB is not involved in the hormonal control of Na^+ transport via ENaC expression. Although the data presented here does not exclude this possibility, both of the studies on *Xenopus* oocytes and Fischer rat thyroid cells showed that PKB could not maintain ENaC activity independently of SGK1 in dexamethasone-stimulated H441 cells (Lee *et al.*, 2007; Diakov *et al.*, 2010).

Rapamycin is known to inhibit TORC1 (Bain *et al.*, 2007; Thoreen & Sabatini; Mansley & Wilson, 2010a), which was crucial for understanding the activation downstream of PI3-kinase. While PI-103 abolishes TORC1 and other protein kinases of the SGK1 pathway (Mansley & Wilson, 2010a; Watt, 2011), rapamycin only causes the inactivation of P70-S6K-Thr^{389}, a TORC1 protein indicator, but no other protein kinases. The inhibition of TORC1 but not TORC2 allows further activation of SGK1 through the phosphorylation of the Ser^{422} residue. PI-103 has been shown to inhibit the dexamethasone-induced Na^+ current but this effect cannot be seen using rapamycin (Watt, 2011). This leads us to conclude that GCs must activate ENaC at the cell surface through a mechanism that depends on PI3-kinase / SGK1 through TORC2, which concurs with previous reports (Blazer-Yost *et al.*, 1998; Record *et al.*, 1998; Alvarez de la Rosa *et al.*, 1999; Paunescu *et al.*, 2000;

Debonneville *et al.*, 2001; Alvarez de la Rosa & Canessa, 2003; Blazer-Yost *et al.*, 2003; Inglis *et al.*, 2009).

4.4.2 Effects of inhibition of the PI3-Kinase / SGK1 pathway on ENaC expression in GC-induced cells.

The initial basis for this study was the findings reported in *Chapter 3* and Watt, (2011) which describe an elevation in ENaC activity when cells are treated for 24 h with dexamethasone. This elevation seems to be driven by the abundance of α-ENaC. All pharmacological inhibitions of the PI3-kinase/TORC2/SGK1 pathway only had a minor effect on α-ENaC in both the total lysates and the surface protein, especially the 95 kDa form, suggesting that the PI3-kinase/TORC2/SGK1 pathway is involved in the control of α-ENaC synthesis (Boyd & Náray-Fejes-Tóth, 2005; Zhang *et al.*, 2007; Reisenauer *et al.*, 2009). However, most of these compounds also inhibit TORC1 so it is interesting that rapamycin, a selective TORC1 inhibitor (Bain *et al.*, 2007), also reduced the abundance of α-ENaC (95 kDa). It is possible that TORC1 may contribute to the control of α-ENaC expression and, in this context, it is relevant that rapamycin has also been shown to disrupt the dexamethasone-induced activation of ENaC in renal epithelia (Mansley & Wilson, 2010a). However, none of the compounds tested in the present study had a discernible effect on the expression of the 75 kDa form of α-ENaC, and the data presented here shows that rapamycin-induced inhibition of TORC1 does not suppress ENaC activity in H441 cells (Watt, 2011). The physiological significance of the effect of the 95 kDa form of α-ENaC is therefore not clear.

In addition, these compounds reduced the expression of γ-ENaC at the cell surface. Since this action was not mimicked by rapamycin, the dexamethasone-induced increase in the

surface expression of this subunit seems to be dependent upon the PI3K – TORC2 – SGK1 pathway. Despite this, there was no effect on β-ENaC. Therefore, PI-103, TORIN1 and GSK650394 clearly abolish the Na^+ current in GC-induced ENaC activity (Watt, 2011), but this cannot be correlated to the withdrawal of ENaC subunits from the plasma membrane.

Whilst dexamethasone clearly activated SGK1, this response was not associated with any change in the activity of TORC2, which is similar to that documented for renal epithelia, where the dexamethasone-induced activation of SGK1 seems to reflect *de novo* synthesis of the SGK1 protein (Wang *et al.*, 2001; Gonzalez-Rodriguez *et al.*, 2007). However, since the nascent protein is catalytically inactive, the PI3K – TORC2 pathway is needed to phosphorylate SGK1-Ser^{422} and confer catalytic activity upon the newly synthesised protein (Kobayashi & Cohen, 1999; Park *et al.*, 1999; García-Martínez & Alessi, 2008; Lu *et al.*, 2010).

Whilst undertaking these studies, we noted that GC-deprived and dexamethasone-stimulated (24 h) cells often appeared to display very similar levels of NDRG1-$Thr^{346/356/366}$ phosphorylation, and this observation (see also Inglis *et al.*, 2009) prompted us to compare the effects of both brief (3 h) and prolonged (24 h) dexamethasone stimulation on the phosphorylation of these residues. These studies confirmed that brief exposure to dexamethasone does increase SGK1 activity without altering the activity of TORC2, but also showed that prolonged (24 h) stimulation had no apparent effect on the activity of either kinase (Watt, 2011). Despite the sustained activation of ENaC, the present data therefore suggest that SGK1 activity returns to its basal level after ~24 h of continuous exposure to dexamethasone. Moreover, experiments that explored the effects of brief (3 h) exposure to dexamethasone on the surface abundance of ENaC subunits showed that the period of increased SGK1 activity was associated with an unambiguous increase in the

surface abundance of α-, β- and γ-ENaC. Furthermore, this response was abolished by GSK650394, indicating that SGK1 does provide a mechanism that allows hormonal control over the surface expression of ENaC subunits. However, despite this clear finding, electrophysiological studies showed that brief (3 h) exposure to dexamethasone did not induce Na^+ currents (Watt, 2011), whilst studies of cells exposed to dexamethasone for ~24 h confirmed the sustained activation of endogenous ENaC as described above.

In conclusion, brief exposure to dexamethasone activated ENaC expression at the cell surface. However, the complex of ENaC subunits detected through Western analysis might not be wholly functional, which may explain the negligible Na^+ current. In addition, these channels may require activation by a secondary mechanism at the plasma membrane (for example, serine-proteases). However, ENaC protein subunits are diminished, especially β- and γ- ENaC, in the presence of an SGK1 inhibitor. This indicates that SGK1 may be an important switch for triggering the hormonal control that governs ENaC abundance on the cell surface. If this is true, it would affect the expression of the ubiquitin ligase protein, Nedd4-2, as this is involved in the final stages of ENaC expression at the cell surface. This leads us to the next chapter, which investigates the effects of dexamethasone on the expression of Nedd4-2.

5

Nedd4-2 modulation through the SGK1 pathway

5.1 Introduction

The GC-induced H441 cells are thought to be regulated through the SGK1 pathway, which in turn activates ENaC at the surface membrane hence stimulating Na^+ transport. The ENaC subunit proteins then need to be withdrawn from the membrane via a mechanism that is dependent on the neural precursor cell-expressed, developmentally down-regulated gene 4 isoform 2 (Nedd4-2), a ubiquitin ligase that binds to proline-rich domains in ENaC subunits and thus targets them for internalisation and degradation. Genetic deletion of Nedd4-2 allows ENaC to remain in the plasma membrane, and this abnormality disrupts lung function by causing excessive Na^+ absorption (Boase *et al.*, 2011; Kimura *et al.*, 2011).

The Nedd4-2 protein consists of four WW domains (Huibregtse *et al.*, 1995; Kamynina *et al.*, 2001a; Asher *et al.*, 2003; Itani *et al.*, 2003) which normally interact with the PY motifs of ENaC (Staub *et al.*, 1997b; Debonneville *et al.*, 2001; Snyder *et al.*, 2002). Due to this interaction, ENaC proteins are ubiquitinated to accelerate the rate of degradation, hence decrementing the channels at the surface membrane which ultimately results in a drastic decrease in the Na^+ current. This has also been observed when Nedd4-2 and ENaC are over-expressed in *Xenopus laevis* oocytes (Malik *et al.*, 2005) and collecting duct cells (Itani *et*

al., 2005), resulting in reduced ENaC activity. This interaction can be reversed by inducing cells with dexamethasone, which is associated with an increase in SGK1, an important molecule for the interaction between the substrate protein ENaC and the ubiquitin protein ligase Nedd4-2.

These findings prompted us to perform subsequent experiments to investigate the association of ENaC-Nedd4-2 in H441 cells. In theory, Nedd4-2 reduces ENaC surface expression, therefore the phosphorylation of Nedd4-2 by protein kinases (eg: in this chapter, SGK1) might hinder the association of ENaC-Nedd4-2 thereby increasing ENaC abundance at the plasma membrane and elevating Na^+ transport. As revealed in *Chapter 4*, ENaC subunit expression on the surface of H441 cells is enhanced by the SGK1 pathway and coincides with ENaC activity (Watt, 2011). Indeed, phosphorylation of the Nedd4-2 protein is affected by SGK1, especially Ser^{327}, Thr^{246} and Ser^{221}, hindering the interaction of Nedd4-2 with the PY-motifs of α-, β-, and γ- ENaC and preventing ENaC from being internalised (Debonneville *et al.*, 2001; Snyder *et al.*, 2002). This raises a pertinent question – does SGK1 directly phosphorylate Nedd4-2 to regulate ENaC? This question is addressed at the end of this chapter.

5.2 Experimental design – aims and objectives

Chapter 4 revealed a transient activation of NDRG1-$Thr^{346/356/366}$ in dexamethasone-treated cells, and increased ENaC expression at the cell surface. This chapter investigates whether SGK1 activity is involved in the phosphorylation of Nedd4-2. Nedd4-2 is thought to be a physiological regulator of ENaC that is essentially regulated downstream of SGK1, inhibiting the ubiquitination-dependent degradation and thus controlling ENaC surface expression (Debonneville *et al.*, 2001; Snyder *et al.*, 2002). As a protein mediator, SGK1 phosphorylates three residues; Ser^{221}, Thr^{246}, and Ser^{327}, to reduce Nedd4-2 binding to

ENaC (Debonneville *et al.*, 2001; Snyder *et al.*, 2002; Zhou & Snyder, 2005). Therefore, I wanted to observe the phosphorylation of Nedd4-2 (Ser^{221}, Thr^{246}, and Ser^{327}) in both GC-induced and GC-deprived H441 cells.

To achieve this, the cell lysates needed to be fully extracted through immunoprecipitation (see a detailed protocol in *Chapter 2:* Materials & Methods) to enable purification of Nedd4-2 from a large amount of lysates. To do this, 1 mg of cell lysates was incubated with the total protein of Nedd4-2 to allow antibody/antigen binding in solution. The protein was then collected using Protein G-coupled agarose beads. This physically isolated the protein of interest from the rest of sample and it was then separated using Western analysis to probe for Ser^{221}, Thr^{246}, and Ser^{327} and total Nedd4-2, respectively.

Since Nedd4-2 consists of multiple isoforms, an experiment was designed to determine a specific protein that corresponds to Nedd4-2 expression. To perform this, 1 mg of protein lysates were incubated with a differential amount of total-Nedd4-2 antibody (0 µg, 1 µg, 3 µg, 5 µg, 10µg) to observe the optimum dosage for Nedd4-2 antibody binding to the protein lysates. Then, a similar experiment was performed with a different amount of protein lysates (0 mg, 0.1 mg, 0.5 mg, 1 mg) and further incubated with an elected amount of total Nedd4-2 (10 µg) to verify protein expression.

Once this had been optimised, the expression of each residue and the total Nedd4-2 protein was further examined. In *Chapter 4*, dexamethasone was shown to transiently increase the phosphorylation of NDRG1-$Thr^{346/356/366}$ and subunits of ENaC (3 h), though this activation was reduced over a longer period (24 h). Therefore, with this data in mind, we chose to investigate whether SGK1 activity correlates with the expression of Nedd4-2. This can be

achieved by inhibition of the SGK1 pathway (PI-103 and GSK650394), as explained in *Figure 5-1*.

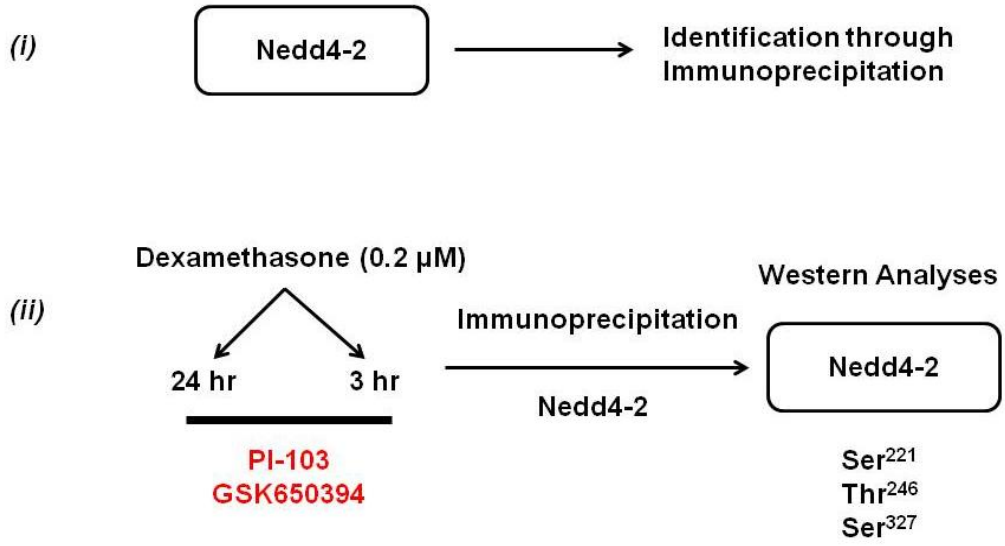


Figure 5-1: A methodology overview of Nedd4-2 expression in human airway cells.

In summary, the aims of this chapter are:

1) To optimise the method of immunoprecipitation prior to Nedd4-2 extraction.
2) To investigate the activation of SGK1-Nedd4-2-ENaC expression in dexamethasone-treated cells at two different time points (3h and 24h).
3) To determine the role of SGK1 in the phosphorylation of Nedd4-2.

5.3 Results

5.3.1 Nedd4-2 verification in H441 cells

H441 cells (1 mg of lysates) were immunoprecipitated against 1, 3, 10 µg of total Nedd4-2 protein. Each sample was electroblotted through 6% SDS polyacrylamide and further probed for total Nedd4-2 antibody. *Figure 5-2A* represents the isoform-binding to the Nedd4-2 antibody. The control sample, containing no protein lysates, expressed nonspecific binding at approximately 80 kDa. Other protein bands also appeared at ~125 kDa and ~105 kDa, which corresponds with other findings shown in *Table 5-1*. These results confirmed that immunoprecipitation works with a large amount of cellular protein (1 mg).

However, Nedd4-2 protein was also observed at approximately 125 kDa, which corresponds to the PC12 cellular extract (Dr. James Hastie, MRC Phosphorylation Unit, University of Dundee; *unpublished data*). Due to this discrepancy, an experiment was conducted to observe whether protein lysates were mass dependent in the presence/absence of the Nedd4-2 antibody. *Figure 5-2B* illustrates that the expression of each Nedd4-2 isoform is elevated with each increment of protein mass, from 0.1 mg to 1 mg of protein lysates. Where the cellular protein is absent, samples containing Sepharose beads expressed a nonspecific binding at ~80 kDa, confirming the expression of the heavy chain of Sepharose G protein beads and thus showing that both isoforms (~125 kDa and ~105 kDa) correspond to Nedd4-2 protein.

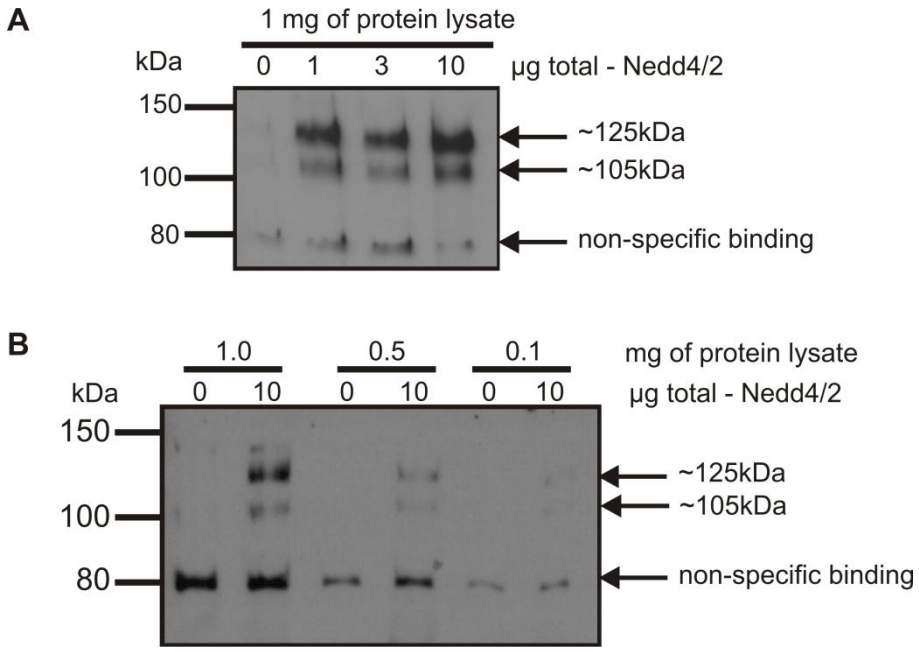


Figure 5-2: Total Nedd4-2 expression in H441 cells extracted through immunoprecipitation.

(A) Antibody mass dependence reveals nonspecific binding and the heavy chain derived from Protein G Sepharose expressing isoforms of total-Nedd4-2. (B) Further experiment to observe whether the protein lysates are mass dependent in the presence/absence of anti-total-Nedd4-2. This confirms the expression of two isoforms of total-Nedd4-2.

Table 5-1: Molecular weight of total Nedd4-2

Authors	Cell Type	Molecular Weight (kDa)
Snyder *et al.*, 2001	COS-7	110
Itani *et al.*, 2005	COS-7	116, 101, 83 (isoforms)
Ichimura *et al.*, 2005	*X. laevis* oocytes	120
Zhou & Snyder, 2005	HEK293T	110
Nagaki *et al.*, 2006	*X. laevis* oocytes	116
Raikwar & Thomas, 2008	HEK293T	116, 101, 83 (isoforms)
Bruce *et al.*, 2008	HEK293T	110
Liang *et al.*, 2010	mpkCCD	100
Hallows *et al.*, 2010	*X. laevis* oocytes	130
Zhang *et al.*, 2010	*X. laevis* oocytes	110

5.3.2 Phosphorylation of endogenous Nedd4-2 in dexamethasone-treated H441 cells

Next, we investigated the phosphorylation of Nedd4-2 in the presence of dexamethasone at 24 h and 3 h. Cells were cultured on a hard surface from day 2 until confluent. 24 h prior to the extraction, a defined media was treated with dexamethasone (0.2 µM) and towards the final 3 h of incubation the other plate was treated with a similar hormone. A control plate of GC-deprived cells was also treated with this hormone. A Bradford assay was conducted to determine 1 mg of cell lysates. The lysates were immuno-precipitated against the total protein, subjected to Western analysis and further probed for three antibodies against the phosphorylated Nedd4-2 (Ser^{221}, Ser^{327}, Thr^{246}), along with Nedd4-2 total protein antibody.

Figure 5-2B shows the significant increase in expression of the three residues of Nedd4-2 (Ser^{221}, Ser^{327}, Thr^{246}), as well as the total Nedd4-2 after a brief (3 h) incubation. However, when the incubation period was extended to 24 h, the expression levels were similar to the control level (*Figure 5-3B*). As anticipated, this finding concurs with the data presented earlier (*Chapter 3, 4*) regarding the activation of SGK1 and ENaC subunits at the cell surface. The mechanism is thought to act rapidly (Staub *et al.*, 1997a; Zhou *et al.*, 2007; Kabra *et al.*, 2008), explained by the increased expression in response to brief exposure to dexamethasone. At the initiation of this project, we found a single protein band for each phosphorylated residue of Nedd4-2. However, as we further optimised the protocol, we noted the expression of two isoforms (~125 kDa and ~105 kDa) that were very similar to the total protein.

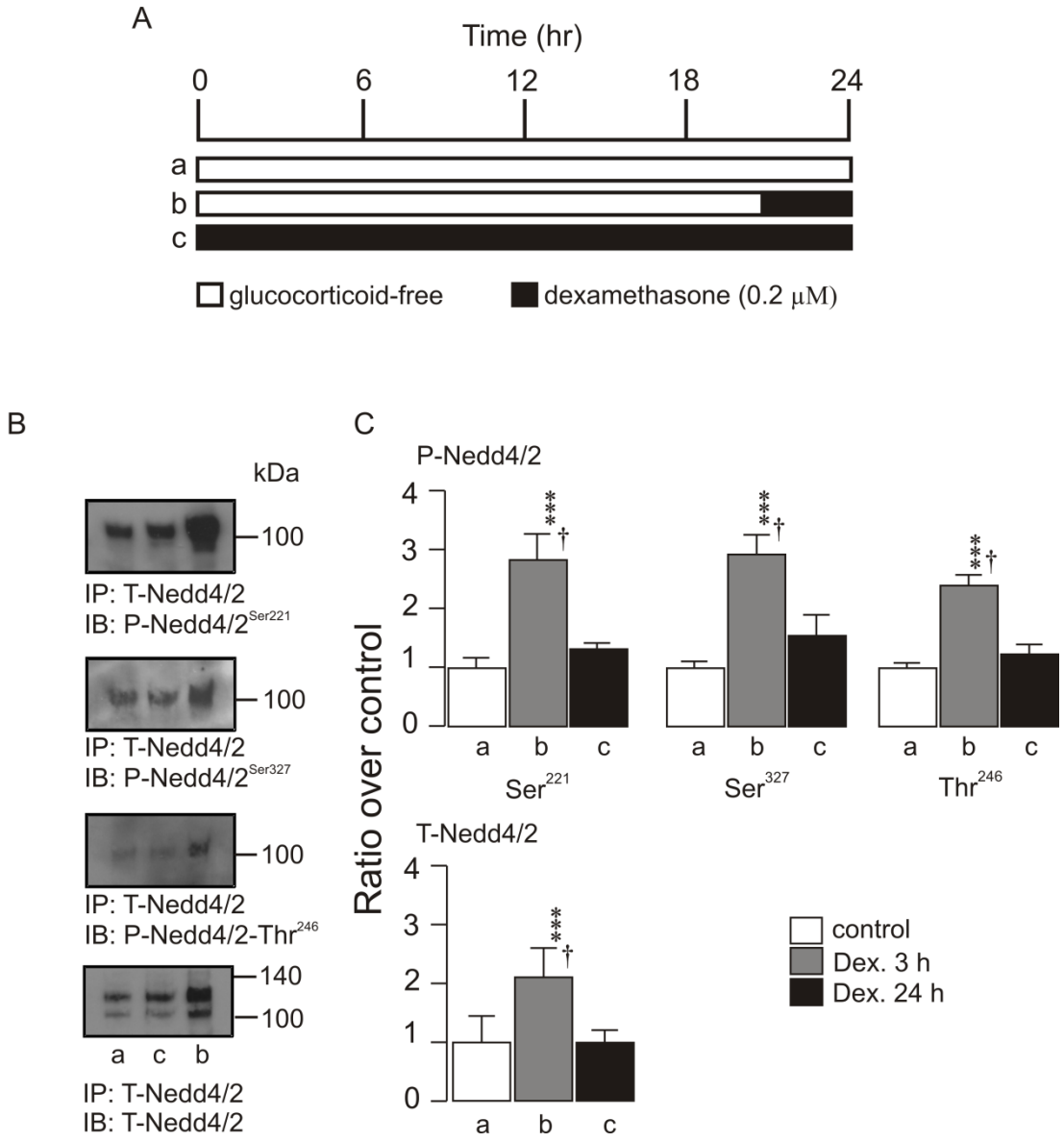


Figure 5-3: The expression of endogenous Nedd4-2 in H441 cells.

(A) Culture conditions 24 h prior to the collection of cell lysates. (B) The blots showing the phosphorylation of Nedd4-2 at three residues (Ser^{221}, Ser^{327}, Thr^{246}) at two different time-points of dexamethasone incubation (20 μM; 24 h and 3 h) as well as total Nedd4-2 expression. (C) Densitometry values of pooled data (n = 8) from (B). Any significant difference to the control is represented by ***p<0.001 whilst †p<0.001 indicates a difference in dexamethasone treatment for 24 h (one-way ANOVA, Bonferroni *post-hoc* test).

5.3.3 Effects of PI-103 on Nedd4-2 in dexamethasone-treated H441 cells

Though previous findings revealed a correlation between the activation of SGK1 and ENaC expression at the cell surface, it was not clear if these phosphorylations were dependent on the PI3-kinase/SGK1 pathway. Using a similar set of experiments to those performed before, the cells were incubated with dexamethasone (0.2 µM) for 24 h and 3 h respectively. GC-deprived control cells were kept in a defined media for 24 h. For the final 3 h of incubation, a pharmacological compound, PI-103, which specifically inhibits PI3-kinase (Mansley & Wilson, 2010a; Watt *et al.*, 2012) was added. The samples were then immunoprecipitated against total Nedd4-2 antibody and later processed through Western analysis and probed for the Ser^{221}, Ser^{327}, Thr^{246} residues of Nedd4-2 and the total protein. *Figure 5-4B* shows an increase in the expression of each residue and the total protein level of Nedd4-2 after brief incubation with dexamethasone, but a reduction in expression to the basal level after 24 h. This confirms the previous findings detailed in *Figure 5-3*.

PI-103 successfully inhibited (if not, reduced) each residue of Nedd4-2 (*Figure 5-4 C, D, E*). However, the inhibition was not clear in GC-deprived cells and cells treated with dexamethasone for 24 h, as any initial expression was not apparent. Interestingly, this effect could not be seen in the total Nedd4-2, as its expression increased despite the inhibition (*Figure 5-4F*). The ultimate effect of this inhibition was shown by cells treated with dexamethasone at 3 h, where the expression was further reduced to the basal level.

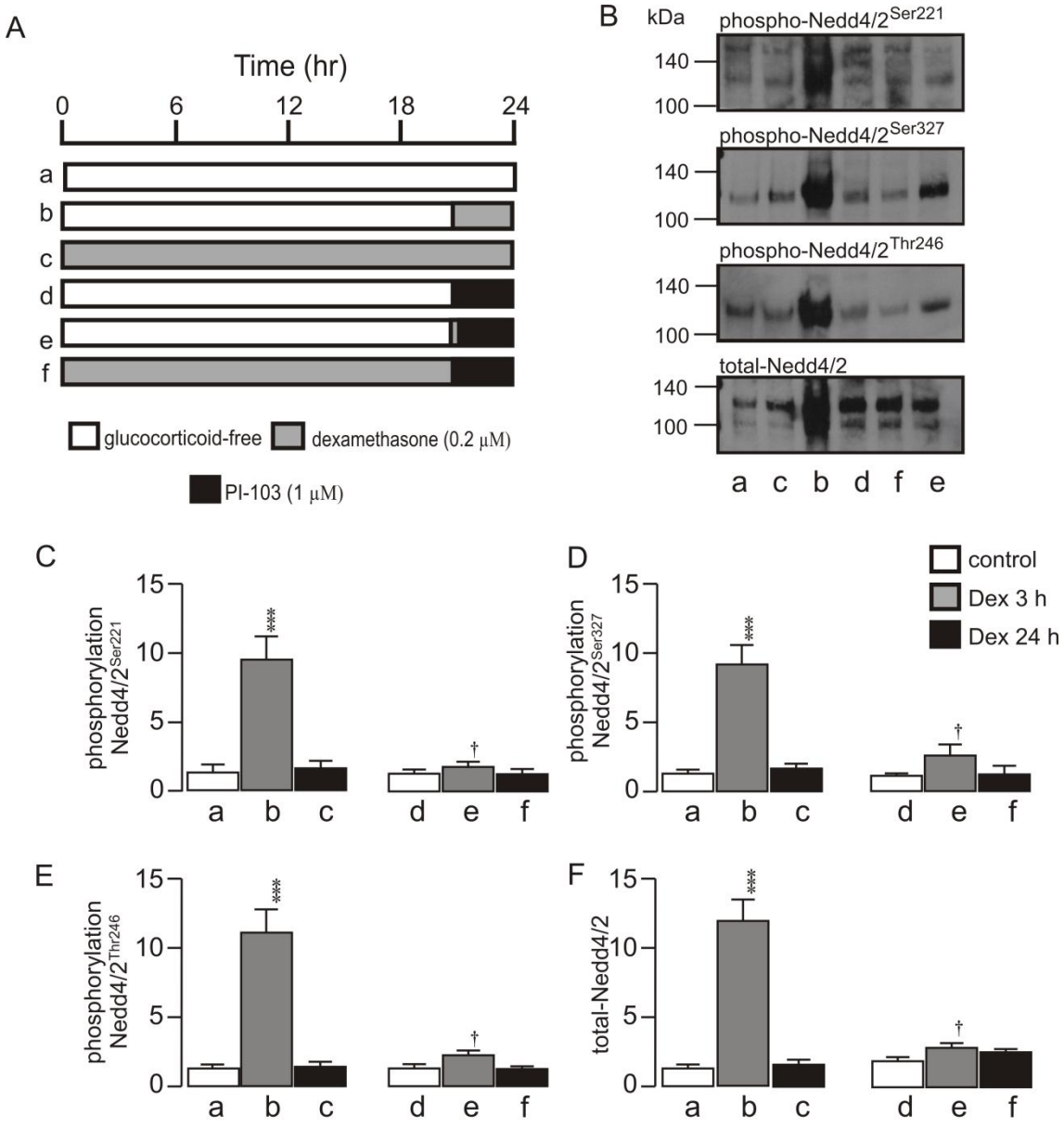


Figure 5-4: Effects of the PI3-kinase inhibitor (PI-103) on phosphorylation of Nedd4-2 in dexamethasone-treated cells.

(A) Culture conditions 24 h prior to the extraction. Western blots in (B) illustrate the expression of phosphorylation sites of (C) Ser^{221}, (D) Ser^{327}, (E)Thr^{246} and (F) total Nedd4-2 in the presence of the PI3-kinase inhibitor, PI103 (1 μM) through immuno-precipitation. These data were pooled (n = 4) and statistically analysed (one-way ANOVA, Bonferroni *post-hoc* test). Asterisks denote significant effects of dexamethasone upon control cells (***p<0.001) whilst the dagger shows the effect of PI-103 on dexamethasone-induced cells (†p<0.001).

5.3.4 Effects of GSK650394 on Nedd4-2 in dexamethasone-treated H441 cells

A specific inhibitor of the SGK1 pathway, GSK650394, successfully inhibited the expression of NDRG1-Thr$^{346/356/366}$ and ENaC at the cell surface, as discussed previously in *Chapter 4*. Therefore, we anticipated a similar effect in this experiment, that the drug would also inhibit the expression of Nedd4-2. As seen in previous experiments, inhibition only has a minor effect on cells that undergo a prolonged treatment with dexamethasone (~24 h), therefore we designed an experiment to assess the effect of this inhibitor on transient dexamethasone-treated cells (3 h).

H441 cells were cultured in a strictly defined medium until confluent. There were three conditions; the GC-free cells, dexamethasone (0.2 µM) induced cells, and GSK650394-treated cells – all treatments were induced approximately 3 h before extraction. These cells were later extracted through immunoprecipitation and subjected to Western analysis.

As anticipated, a brief period of dexamethasone incubation enhances the expression of Nedd4-2 residues and total protein. *Figure 5-5B* illustrates the dramatic increase in expression of these residues in dexamethasone-treated cells. The inhibitor also reduced the expression in GC-deprived cells (*Figure 5-5C, D, E*). Interestingly, for total Nedd4-2 (*Figure 5-5F*) GSK650394 does not completely inhibit GC-free cells but does reduce expression to a point close to that of the basal expression.

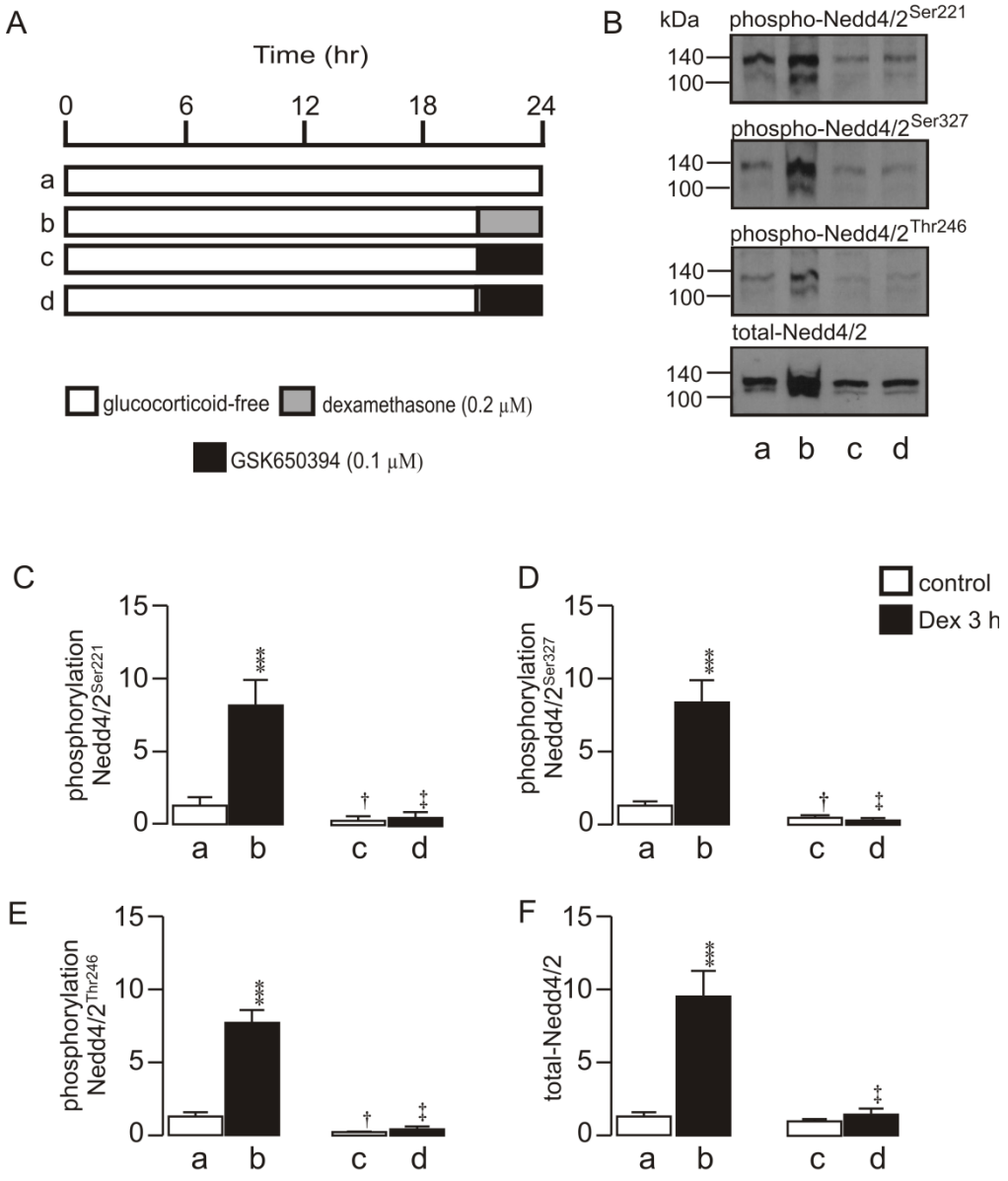


Figure 5-5: Effects of the SGK1 inhibitor, GSK650394, on the phosphorylation of Nedd4-2 in dexamethasone-treated cells.

(A) Culture conditions 24 h prior to the extraction. Western blots in (B) represent the effect of the SGK1 inhibitor, GSK650394, on the phosphorylation of Nedd4-2 (Ser^{221}, Ser^{327}, Thr^{246}) as well as total protein. (C), (D), (E), (F) Bar graphs showing the densitometry value of pooled data (n = 4) and asterisks indicate a significant difference between dexamethasone-treated cells and control cells (***p<0.001). A dagger shows the effect of GSK650394 on control cells (†p<0.05) and double daggers indicate the effect of GSK650394 on dexamethasone-treated cells (‡p<0.001) (one-way ANOVA, Bonferroni *post-hoc* test).

5.4 Discussion

Nedd4-2 is a member of the Nedd4 family, which is similar to the E6-Associated Protein C-Terminus (HECT) domain type E3 ubiquitin protein ligase that is widely expressed in mammalian tissues and cells. This protein is important because it assists various cellular functions, from regulation of transcription to intracellular trafficking, as well as protein degradation (Rotin *et al.,* 2000) which involves multiple WW domains. A number of *in vitro* binding studies have suggested that different WW domains produce various affinities against the PY motif of ENaC (Kamynina *et al.,* 2001a; Fotia *et al.,* 2003; Henry *et al.,* 2003; Itani *et al.,* 2003; Bhalla *et al.,* 2005).

Nedd4-2 appears to comprise a higher affinity for ENaC binding than the Nedd4 protein. This is due to the high number of consensus SGK1 phosphorylation sites that specifically bind to the WW domains of Nedd4-2 (Debonneville *et al.,* 2001; Snyder *et al.,* 2002). The form of Nedd4-2 found in human cells (hNedd4-2) reportedly exists in multiple isoforms, which possibly arose from alternate transcription and translation due to the variable splicing of some internal exons (Itani *et al.,* 2003). These domains are approximately 40 amino acids in length and are located in the WW domains of Nedd4-2 to mediate the interaction of SGK1-Nedd4-2. Whilst some isoforms involved in the phospho-binding domain may dictate membrane localisation (Plant *et al.,* 1997), the others have a strong affinity for SGK1 phosphorylation sites (Itani *et al.,* 2003). Out of all the four WW domains of hNedd4-2, WW 3 and 4 are proposed to bind strongly to the PY motif of ENaC whilst WW 1 and WW 2 have little or no affinity binding (Itani *et al.,* 2003).

In theory, Ser^{221} and Thr^{246} are found between WW domains 1 and 2 while Ser^{327} is located between WW domains 2 and 3 (Synder *et al.,* 2002). Increased expression of Ser^{327} is thought to be the most crucial for Nedd4-2 activation and this is further elevated by Ser^{221}

and Thr^{246} to achieve maximum activity. Snyder *et al.*, (2004a) have suggested that only Ser^{327} and Thr^{246} potentiate the binding of SGK1 to inhibit binding to ENaC, however this does not concur with the data presented in this chapter. Although all three phosphorylation sites of Nedd4-2 are successfully expressed in conjunction with SGK1 activity, it is worth noting that SGK1 activation is absolutely essential for ENaC regulation and this must be mediated by Nedd4-2 (*Figure 5-5*). In addition, SGK1 itself can stimulate ENaC at the cell surface without phosphorylating Nedd4-2 (Wang *et al.*, 2001), mainly through the activation of α-ENaC (Diakov & Korbmacher, 2004), as this subunit contains a possible activation site at the promoter region that can be stimulated by SGK1 (McTavish *et al.*, 2009).

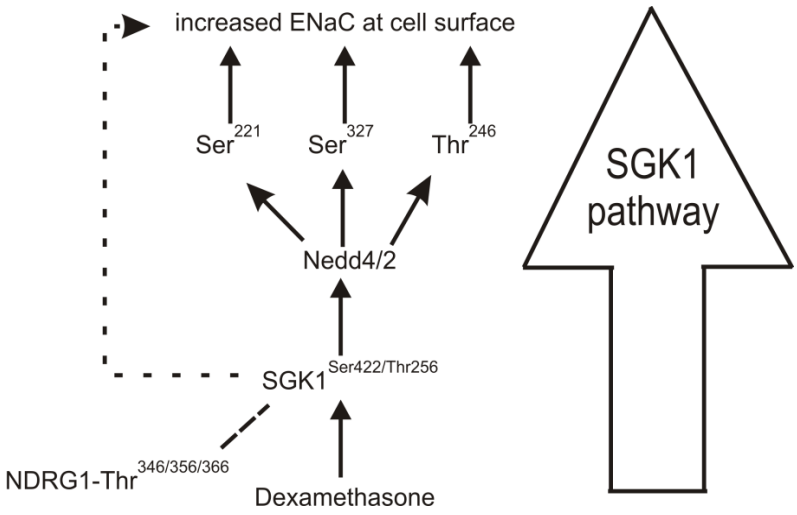


Figure 5-6: A proposed model for endogenous ENaC modulation through the SGK1 pathway involving the phosphorylation of Nedd4-2.

Dexamethasone has been shown to increase SGK1 activity transiently (3 h - 6 h) but the expression is reduced to the basal level after 24 h. This was anticipated in the previous chapter and also coincides with the findings observed by Itani *et al.*, (2005); Inglis *et al.*, (2009); Mansley & Wilson, (2010a) and Watt *et al.*, (2012). The brief effect of

dexamethasone on the SGK1 protein indicator (i.e., P-NDRG1-Thr$^{346/356/366}$) therefore provides an attractive model showing the activation of endogenous Nedd4-2 and thus the regulation of ENaC at the cell surface. Total Nedd4-2 expression was elevated after 3 h of dexamethasone treatment but returned to normal after prolonged treatment (24 h). Interestingly, three residues of Ser-Thr in Nedd4-2 also increased due to the effects of dexamethasone (*Figure 5-6*), possibly due to the augmentation of total protein.

The activation of phosphorylation sites of Nedd4-2 is thought to correspond well with the overall expression of the total protein, leading to a functioning Nedd4-2 and thus a decrease in the number of ENaC channels and reduction of epithelial Na^+ absorption. Many studies have shown that this process occurs rapidly, consistent with the data in this chapter (Staub *et al.*, 1997a; Zhou *et al.*, 2007; Kabra *et al.*, 2008). A recent study by Chandran and colleagues investigated heterologously expressed *Xenopus* Nedd4-2 and found that Nedd4-2 degradation is prevented by the phosphorylation of a residue equivalent to Ser^{327} in the human protein (Chandran *et al.*, 2011). As far as we are aware, the present study is the first to explore the effect of agents that control Na^+ transport (e.g., dexamethasone) which might modulate the expression / phosphorylation of endogenous Nedd4-2. Studies by Chandran *et al.*, (2011) have noted that physiological stimuli that evoke phosphorylation of this residue can stabilise the protein and increase its abundance, an effect which may well explain the present findings in this chapter. Moreover, it has been suggested that such control over Nedd4-2 expression may provide a novel aspect to the mechanism that allows the hormonal control of Na^+ absorption; the data presented here are consistent with this hypothesis.

Therefore, these findings support the idea that Nedd4-2 can play a role in maintaining ENaC at the cell surface. This mechanism may be crucial for preventing build up of excessive Na^+ in the plasma membrane. The targeted disruption of Nedd4-2 in the mouse has been shown to cause hypertension as result of the excessive Na^+ intake (Shi *et al.*,

2008). Nedd4-2 can be switched off to maximise ENaC activity and the mechanism is assisted by phosphorylation through Ser-Thr, as presented in this chapter.

The effect of Nedd4-2 on ENaC expression at the cell surface also correlates with an increase in SGK1 activity. However, this theory contradicts other studies by Itani *et al.*, (2005) and Zhou & Snyder, (2005) where a reduced level of Nedd4-2 was linked to an increment in the SGK1 level. The mechanism was also shown to be reversible; where Nedd4-2 was reactivated in a low level of SGK1 (Itani *et al.*, 2005; Zhou & Snyder, 2005). The discrepancy is due to the over-expression of Nedd4-2 in the cells where SGK1 has a synergistic effect controlling the excess production of Nedd4-2. In contrast, in this project all samples were derived from the endogenous cellular extract (as opposed to an over-expression experiment) which was better for investigating the relationship between SGK1 and Nedd4-2.

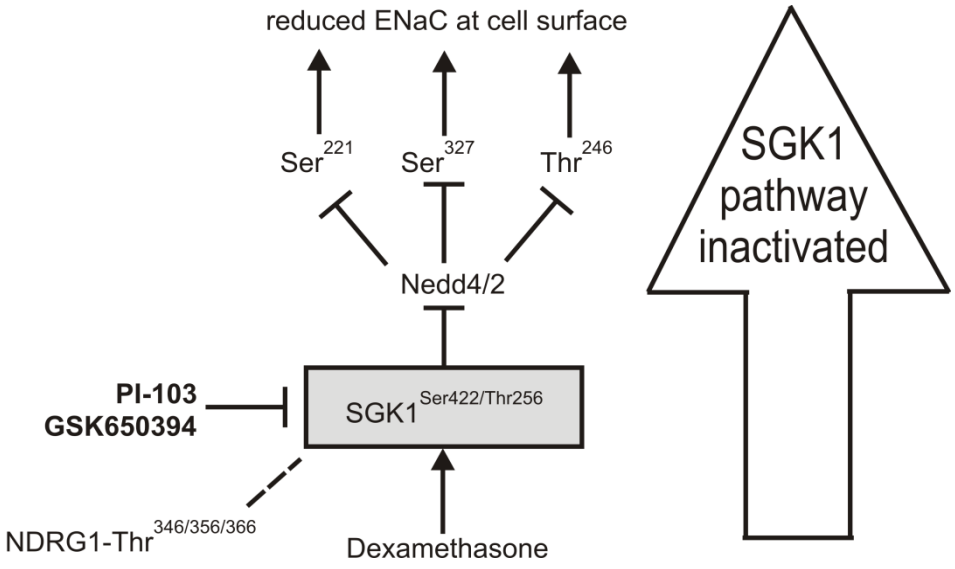


Figure 5-7: SGK1 pathway inactivation leads to the inhibition of residues of Nedd4-2 and thus increases rapid degradation of ENaC.

SGK1 activity was revealed by inducing cells with PI-103 and GSK650394, which successfully silenced the pathway (*Chapter 4*). *Figure 5-7* depicts the role of these drugs on the SGK1 pathway. They act by preventing the interaction between SGK1 and Nedd4-2, leading to increased internalisation of ENaC. Therefore, SGK1 inhibition stops the PY motif binding to the phosphorylated residues of Nedd4-2, as demonstrated in this chapter. As a result, Nedd4-2 rapidly degrades ENaC at the cell surface (*Figure 5-7*), especially the β- and γ-ENaC subunits (see reduction of these subunits as a result of GSK650394 treatment in *Chapter 4*).

The reduction of both β- and γ- ENaC indicates the importance of Nedd4-2 which is linked to Liddle's Syndrome, caused by deletion or alteration of the PY motif in β- and γ- ENaC (Staub *et al.*, 1996; Goulet *et al.*, 1998). This results in an increase in ENaC at the cell surface due to the inability of Nedd4-2 to internalise ENaC from the membrane (Harvey *et al.*, 2001; Kamynina *et al.*, 2001c). The mechanism also leads to increased renal Na^+ absorption which is associated with hypertension, as a result of a mutation at the C-terminus of the PY motif of β- and γ- ENaC (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a; Hansson *et al.*, 1995b).

In conclusion, the endogenously expressed hNedd4-2 protein coincides with the transient activation of SGK1 in H441 cells, to further elevate ENaC at the cell surface in the presence of the SGK1 stimulator, dexamethasone. This effect is reduced in the presence of the PI3-kinase downregulated SGK1 pathway inhibitor (PI-103) and GSK650394 which specifically inhibit the SGK1 protein. Whilst both β- and γ- ENaC were abolished in the absence of SGK1, these compounds only had a minor effect on the expression of α-ENaC (especially GSK650394, see *Chapter 4*), suggesting that there may be an independent mechanism that stimulates subunit expression outside of the SGK1 pathway. One possible mechanism

would be a direct α-ENaC activation by SGK1 (Wang *et al.*, 2001; Diakov & Korbmacher, 2004) which might not involve Nedd4-2. Others have proposed that ENaC can be stimulated by PKA (Snyder *et al.*, 2004a), which is explored in the next chapter: investigating the regulation of ENaC by the PKA-activating pathway.

6

Regulation of ENaC and Nedd4-2 by the PKA pathway

6.1 Introduction

The data presented in this thesis supports the theory that GC-induced expression of ENaC at the surface of H441 cells can be regulated via the SGK1 pathway. SGK1 phosphorylates the endogenous ubiquitin ligase Nedd4-2 to prevent internalisation of ENaC from the cell surface; however, others have proposed that the cAMP activating protein kinase A (PKA) pathway could modulate ENaC through a similar mechanism (Debonneville *et al.*, 2001; Snyder *et al.*, 2002; Snyder *et al.*, 2004a). The most notable theory was reported by Snyder (2000) when investigating the role of cAMP in Liddle's syndrome. This study found that cAMP mediated the translocation of ENaC towards the cell surface, but that this was then disrupted by the mutation of the cytoplasmic COOH-termini of the β- and γ- ENaC subunits (Snyder, 2000). A subsequent study by the same group proposed that the cAMP activating PKA pathway interacts with residues of Nedd4-2 in the SGK1 pathway (Snyder *et al.*, 2004a) prior to ENaC subunit expression at the cell surface. The physiological role of Nedd4-2 is to limit the rate of Na^+ absorption (Snyder *et al.*, 2002; Snyder *et al.*, 2004a; Snyder *et al.*, 2004b), this ubiquitin ligase can be phosphorylated, and thus inactivated, by protein kinase A (PKA) and serum and GC-inducible kinase 1 (SGK1). Moreover, since the activities of these regulatory kinases are under the control of hormones / neurotransmitters in the extracellular space, the regulated inactivation of Nedd4-2 is thought to contribute to

the hormonal control of Na^+ absorption by allowing α-, β- and γ-ENaC to remain at the cell surface, an event that would lead to increases Na^+ transport by increasing the membrane's permeability to Na^+ (Snyder *et al.*, 2002; Flores *et al.*, 2005; Debonneville *et al.*, 2001; Lang *et al.*, 2006; Snyder, 2005).

GC hormones are physiologically-important regulators of pulmonary Na^+ transport. Dexamethasone, a synthetic GC, can evoke ENaC-dependent Na^+ currents in H441 human airway epithelial cells via an SGK1-dependent mechanism. Moreover, these GC-induced Na^+ currents can be augmented by agents that activate PKA (Murray *et al.*, 2005; Thomas *et al.*, 2004; Ramminger *et al.*, 2004; Clunes *et al.*, 2004; Vasquez *et al.*, 2008) and these findings are therefore in accordance with the conceptual model outlined above. However, dexamethasone-induced SGK1 activity in these cells peaks after ~3 h of stimulation and then declines to its basal value within ~24 h. Moreover, only minor changes to the surface abundance of α-, β- and γ-ENaC persist in cells that have been exposed to dexamethasone for ~24 h (*Chapter 3*), and the dexamethasone-induced Na^+ currents are well maintained (Watt *et al.*, 2012). Despite the evidence implicating the SGK1 – Nedd4-2 signalling pathway in the control of Na^+ absorption, it is not easy to see how this hypothesis can explain the persistent activation of ENaC activity seen in GC-treated human airway epithelial cells (Watt *et al.*, 2012). In order to clarify the mechanisms that allow signalling via PKA / SGK1 to control ENaC activity in these cells, we used phospho- specific antibodies to explore the effects of these kinases upon the phosphorylation / expression of endogenous Nedd4-2, whilst parallel analyses of surface-exposed proteins monitored changes to the amounts of α-, β- and γ-ENaC present in the plasma membrane.

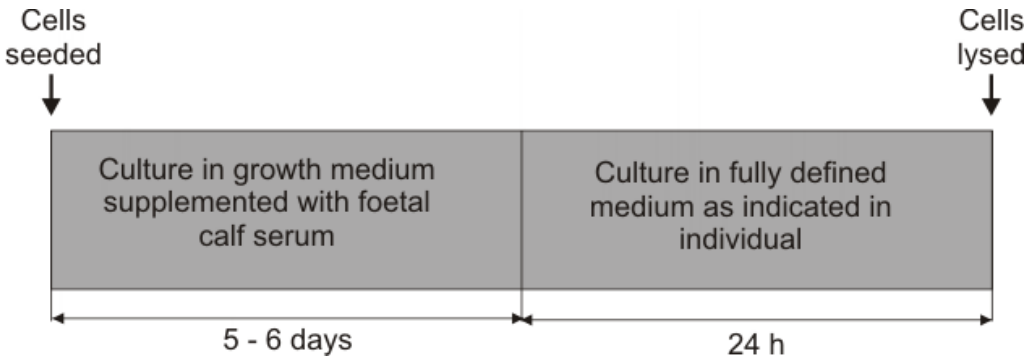
6.2 Experimental design - aims and objectives

As seen previously in *Chapter 5*, three residues of Nedd4-2 (Ser^{221}, Ser^{327}, and Thr^{246}) were transiently phosphorylated in the presence of the GC hormone. In theory, this would lead to a failure in PY motif binding to ENaC and thus increase ENaC expression at the cell surface. Therefore, in this chapter, further examination into the acute effect of cAMP agonists towards Ser-Thr residues of Nedd4-2 is conducted, in addition to investigating the abundance of ENaC in the surface pool of proteins. This idea was prompted by previous data from our lab (Clunes *et al.*, 2004; Inglis *et al.*, 2009) which reported a natriferic response in H441 cells that had been treated with an acute cAMP agonist cocktail after prolonged exposure to dexamethasone (~ 24 h; Woollhead & Baines, 2006).

First, we needed to monitor PKA activity, which can be observed using CREB-Ser^{133}, a substrate that indicates the activation of the cAMP-dependent PKA protein messenger. Concurrently, we also needed to monitor the effects of cAMP agonists on the expression of NDRG1-$Thr^{346/356/366}$, a specific protein indicator for SGK1. Then, a time-dependent experiment was performed to determine when the protein substrates of PKA were activated. At the same time, similar experimental methods were applied to the target of Nedd4-2 residues. Protein lysates were extracted using an immunoprecipitation protocol with an antibody for Nedd4-2 and then probed for the protein residues as well as the total protein. Next, ENaC subunit proteins were extracted using biotin-labelled surface cells treated with dexamethasone and cAMP agonists. The inhibition of SGK1 activity using GSK650394 allowed us to predict the actual effect of cAMP agonists on the abundance of ENaC and the phosphorylation of Nedd4-2.

The cells were cultured using the standard procedure detailed in the Materials & Methods (*Figure 6-1*). A set of experiments were designed to test the acute effect of cAMP agonists

that contained 10 µM forskolin, 100 µM isobutylmethylxanthine, 1 mM N^6, 2'-*O*-dibutyryladenosine 3'5'-cyclic monophosphate for 20 mins (Clunes *et al.*, 2004) or/and dexamethasone (0.2 µM) treatment for 24 h and 3 h, respectively. The cAMP agonists were added for the final 20 mins of the experiment. Hormone-deprived cells were maintained in a fully defined medium for the final 24 h prior to extraction. The SGK1 inhibitor (GSK650394, 0.1 µM) was added in the final 3 h of the experiment to the dexamethasone or cAMP agonist incubation. A combination of the techniques used in *Chapters 4* and *5* (*i.e.*, cell surface biotinylation and immunoprecipitation) were carried out, then the proteins were processed using Western analysis. We probed for subunits of ENaC (α-, β-, γ-) and residues of Nedd4-2 (Ser^{221}, Ser^{327}, Thr^{246} and total protein). Incubation with the endogenous protein kinases CREB-Ser^{133} and NDRG1-$Thr^{346/356/366}$ served a complementary test in this chapter to indicate the activation of PKA and SGK1, respectively.



Analytical methods	
SGK1 activity	NDRG1-$Thr^{346/356/366}$ phosphorylation
PKA activity	CREB-Ser^{133} phosphorylation
Nedd4/2 phoshorylation	IP / Western blot
ENaC surface abundance	Western blotting of surface-exposed proteins

Figure 6-1: An overview of the experimental design used throughout the chapter.

The objectives of this chapter can be summarised as follows:

1) To observe the expression of PKA/SGK1 in cAMP-induced H441 cells.

2) To determine the effect of cAMP agonists on Nedd4-2 and the abundance of ENaC at the cell surface.

3) To establish the role of cAMP agonists in dexamethasone-induced cells on Nedd4-2 and the abundance of ENaC at the cell surface.

6.3 Results

6.3.1 Cyclic AMP induced activation of PKA and SGK1

Exposing GC-deprived cells to a cocktail of cAMP agonists, which are designed to promote activation of cAMP-dependent signalling pathways, increased the abundance of the Ser^{133}-phosphorylated CREB without altering the overall CREB expression level (*Figure 6-2B*). These drugs therefore promote phosphorylation of CREB-Ser^{133} and, since this residue is an archetypal PKA substrate, it is clear that PKA becomes active under these conditions. The response peaked within ~20 mins and, although there was some decline from this maximum, the response persisted for at least 24 h (*Figure 6-2B,C*). The cAMP agonists also evoked phosphorylation of NDRG1-$Thr^{346/356/366}$ (*Figure 6-2D,E*) and, since these residues are phosphorylated by SGK1 and not by other, closely related kinases (Murray *et al.*, 2004; Murray *et al.*, 2005), SGK1 must also become active under these conditions. This response, in contrast to the activation of PKA, did not become apparent until ~ 2 h and had elapsed and peaked after ~12 h (*Figure 6-2D, E*).

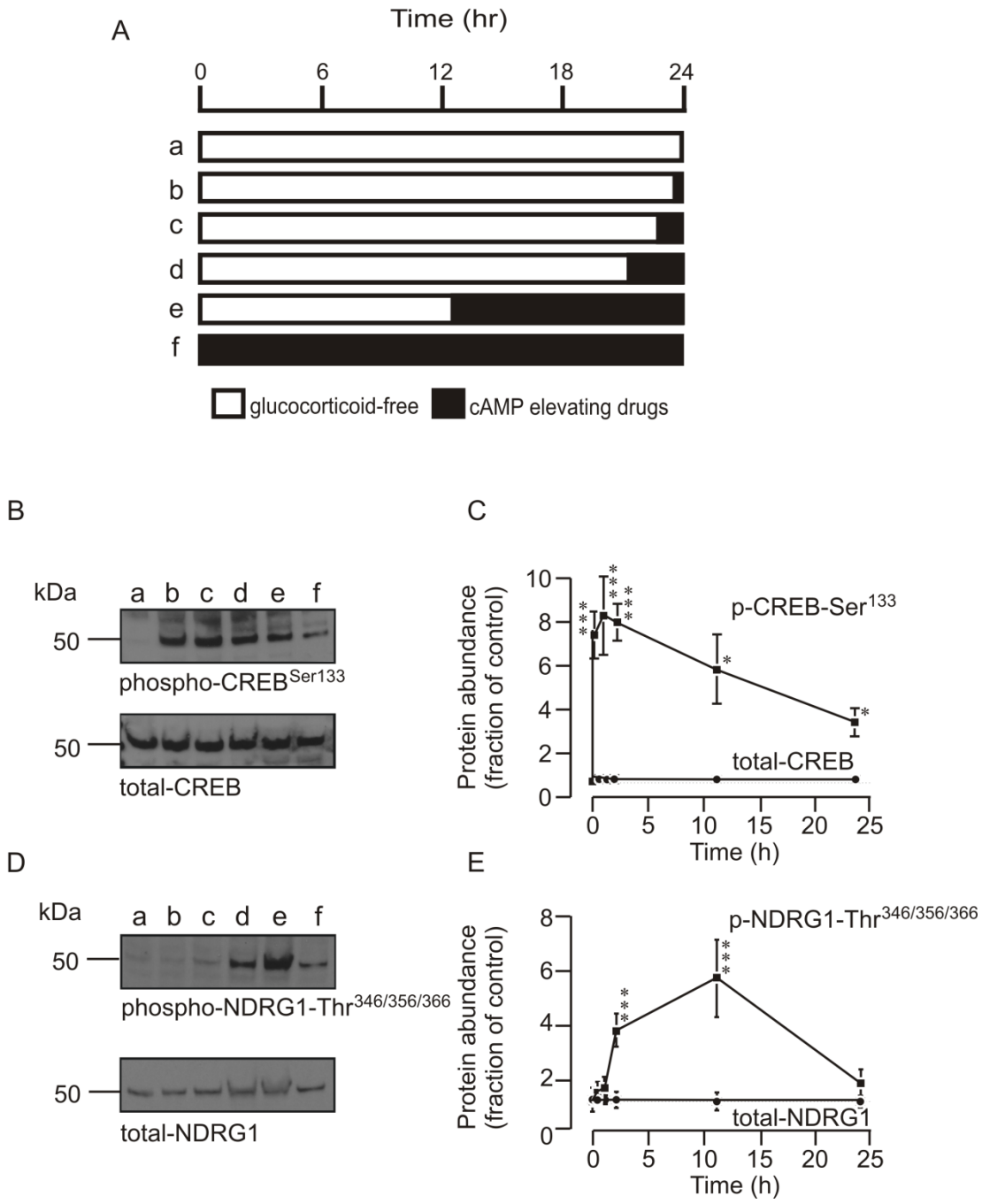


Figure 6-2: cAMP agonists phosphorylate the endogenous protein kinase PKA and SGK1 pathways

(A) H441 cells were prepared as indicated. Typical Western blots show the effects of cAMP elevating drugs on the abundance of (B) phosphorylation of CREB-Ser^{133} (upper panel), total protein (lower panel) and (C) phosphorylation of NDRG1-Thr$^{346/356/366}$ (upper panel) and total protein (lower panel) initially at 20 mins for a 24 h period of incubation. The expression of both phosphorylated and total proteins are presented in the pooled data (n = 3) which indicates significant effects of exposure compared to unstimulated cells. *p<0.05, ***p<0.001 (one-way ANOVA, Bonferroni *post-hoc* test).

6.3.2 cAMP agonists phosphorylate Nedd4-2 and the total protein

The immunoprecipitation protocol (see Materials & Methods) allowed the recovery of two proteins with molecular weights of ~105 kDa and ~125 kDa, which is consistent with the expression of Nedd4-2 (Boase *et al.*, 2011). Preliminary experiments verified that the isolation of these proteins was dependent on the inclusion of the Nedd4-2 antibody in the immunoprecipitation reaction. To explore the extent to which Nedd4-2 is phosphorylated at each of three physiologically-relevant (Snyder *et al.*, 2004; Debonneville *et al.*, 2001) sites (Ser^{221}, Ser^{327} and Thr^{246}), proteins immuno-purified in this way were subjected to Western analysis using phospho-specific antibodies directed against these residues (*Figure 6-3B*). Exposing GC-deprived cells to cAMP agonists increased the abundance of the Ser^{221}- (*Figure 6-3C*) and Ser^{327}- (*Figure 6-3D*) phosphorylated forms of Nedd4-2 and, initially, these responses followed a time course very similar to the activation of PKA. Although increased abundance of Thr^{246}-phosphorylated Nedd4-2 also occurred, this did not become apparent until ~2 h had elapsed and reached a peak after ~12 h (*Figure 6-3E*). However, despite these initial effects, prolonged exposure to cAMP agonists eventually caused a marked fall in the abundance of each phospho-protein, and phosphorylated Nedd4-2 thus became barely detectible in cells that had been exposed to these drugs for 12 – 24 h (*Figure 6-3 C, D, E*). Western analysis of immunoprecipitated proteins using the antibody against full length Nedd4-2 showed that the cAMP agonists also caused an initial increase in the overall abundance of this protein and this response, in common with the effects on the $Ser^{221/327}$-phosphorylated forms of Nedd4-2, initially followed a time course similar to the activation of PKA (*Figure 6-3F*). However, prolonged exposure to cAMP agonists caused a marked fall in Nedd4-2 expression and this protein also became virtually undetectable after 12 – 24 h (*Figure 6-3F*). These experiments were undertaken by stripping / re-probing blots that had initially been probed with a phospho-specific antibody and we were therefore concerned that the apparent changes in overall abundance may be artefacts caused by the

incomplete removal of the phospho-specific antibodies from the blots. This possibility was, however, discounted by additional experiments in which the blots were initially probed with the antibody against full length Nedd4-2. The data obtained in this way were virtually identical to those described above, and it is therefore clear that the cAMP agonists cause biphasic changes to the Nedd4-2 expression level in GC-deprived cells.

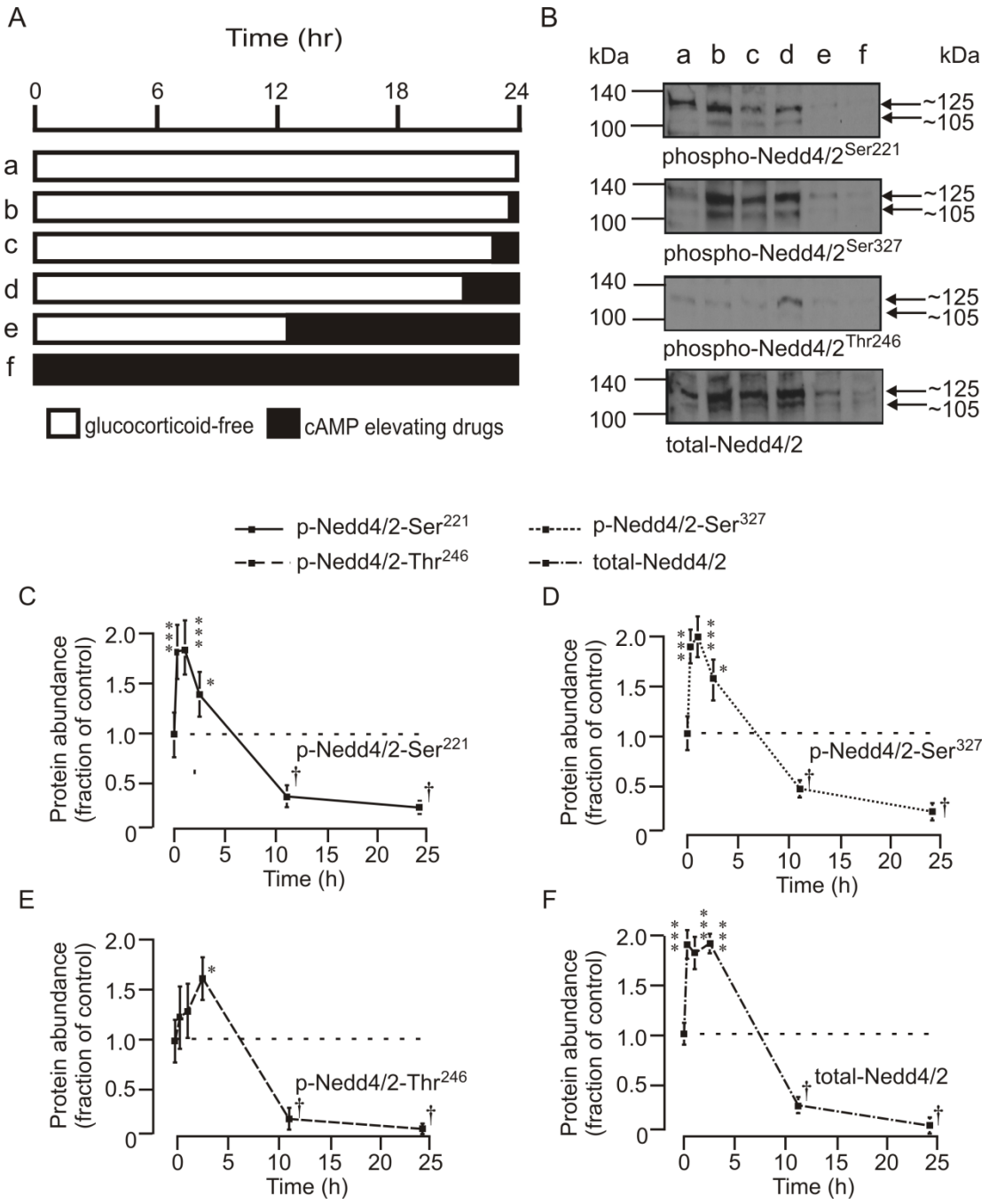


Figure 6-3: Time-course dependence of cAMP agonists on the phosphorylation of Nedd4-2 (Ser^{221}, Ser^{327}, and Thr^{246}) and total Nedd4-2 protein.

(A) The experimental design was applied to H441 cells as indicated. Cells were precipitated against total Nedd4-2 and probed for (B) three residues of Nedd4-2 (Ser^{221}, Ser^{327}, and Thr^{246}) as well as the total protein. Figures in C, D, E and F represent the densitometry value of the protein against the duration of incubation (h) as shown in (B) to indicate activation over time. *p<0.05 and ***p<0.001 denote a significant increase in the effect of cAMP agonists whilst the dagger (†) p<0.05 represents a significantly decreased effect compared to control cells (one-way ANOVA, Bonferroni *post-hoc* test).

6.3.3 Direct comparison of the effects of cAMP / dexamethasone

Exposing GC-deprived cells to cAMP agonists for 20 mins in order to activate PKA and not SGK1 increased the abundance of the Ser^{221}-phosphorylated, Ser^{327}-phosphorylated and total forms of Nedd4-2 but did not alter the abundance of the Thr^{246}-phosphorylated protein. This response is essentially identical to that described above, and parallel studies of cells acutely (3 h) exposed to dexamethasone in order to activate SGK1 independently of PKA, confirmed that this stimulus also causes a clear increase in the abundance of the Thr^{246}-phosphorylated Nedd4-2 (*Figure 6-4*). Further experiments used this protocol to compare the effects of selectively activating PKA and SGK1 on the surface abundance of the ENaC subunits. Data from unstimulated cells confirmed the pattern expression described above and, as anticipated, activating SGK1 increased the surface abundance of α-, β- and γ-ENaC (*Figure 6-5*). Activating PKA (i.e., cAMP agonists for 20 mins), on the other hand, increased the amount of α-ENaC in the membrane (*Figure 6-5C*) but had no effect on β- and γ-ENaC (*Figure 6-5D,E*). Activation of SGK1 and PKA in GC deprived cells therefore has different effects on the phosphorylation / abundance of Nedd4-2, and on the surface expression of ENaC subunits.

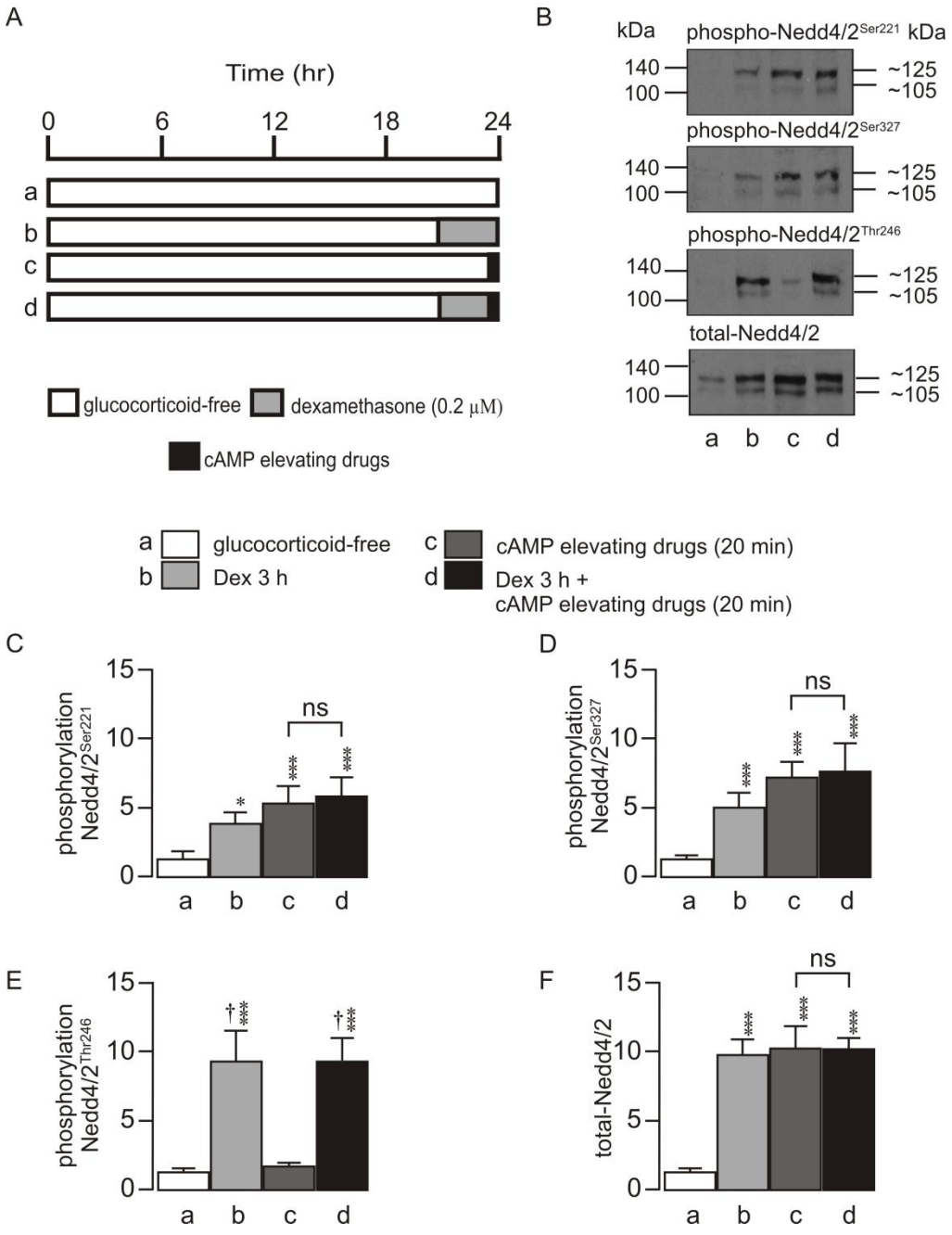


Figure 6-4: The response of Nedd4-2 expression to cAMP agonists in the presence of transient dexamethasone-treated cells.

(A) Three independent sets of cells were treated as dexamethasone-deprived. Cells were exposed to dexamethasone for 3 h and cAMP agonists were added for the final 20 mins of incubation. (B) These cells were probed for three phosphorylated Nedd4-2 residues (Ser^{221}, Thr^{246}, and Ser^{327}) and total Nedd4-2. Densitometry values were determined from the blots and depicted as in C, D, E, F. Asterisks denote statistically significant effects towards hormone deprived cells (control), $*p<0.05$, $***p<0.001$. The dagger shows a significant increase resulting from the effects of cAMP elevating drugs, $\dagger p<0.001$ (one-way ANOVA, Bonferroni *post-hoc* test).

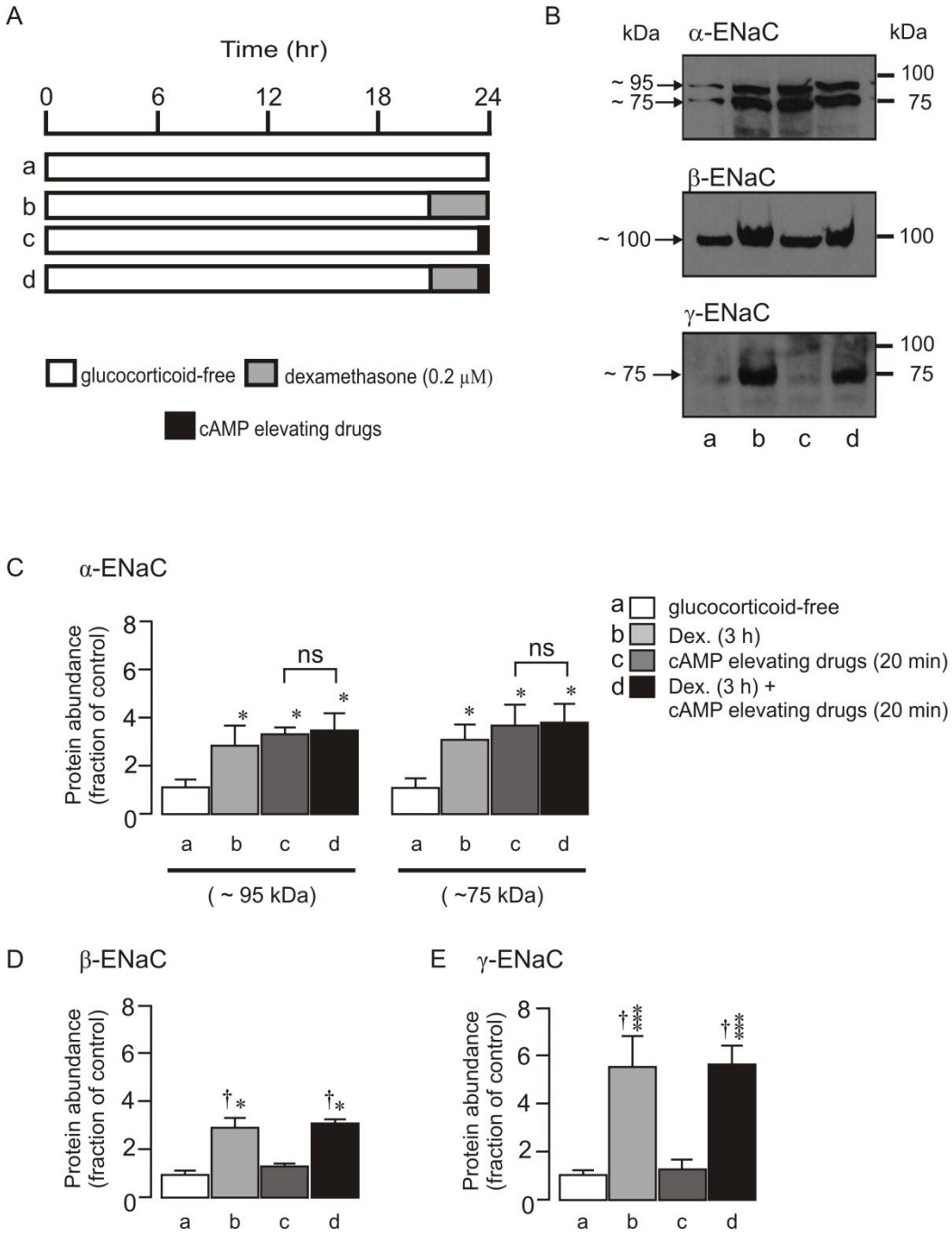


Figure 6-5: The abundance of ENaC at the cell surface in response to cAMP agonists in the presence of transient dexamethasone-treated cells.

(A) Three independent sets of cells were treated as dexamethasone-deprived. Cells were exposed to dexamethasone for 3 h and cAMP agonists were added for the final 20 mins of incubation. (B) These cells were probed for α-ENaC, β-ENaC and γ-ENaC from the cell surface protein pool. Densitometry values were determined from the blots and are depicted as in C, D, and E. Asterisks denote statistically significant effects from the hormone deprived cells (control), *$p<0.05$, ***$p<0.001$. The dagger shows a significant increase resulting from the effects of cAMP elevating drugs, †$p<0.05$ (one-way ANOVA, Bonferroni *post-hoc* test).

6.3.4 Effects of cAMP agonists on dexamethasone-treated cells

Cells that had been stimulated with dexamethasone (0.2 μM) for 24 h and the cAMP agonists (20 min) increased the abundance of the Ser^{221}- and Ser^{327}-phosphorylated Nedd4-2 (*Figure 6-6C,D*) and also increased the overall Nedd4-2 expression level (F*igure 6-6F*). Whilst these responses resembled those seen in dexamethasone-treated cells (see above), the cAMP agonists did not increase the abundance of Thr^{246}-phosphorylated Nedd4-2 in dexamethasone-treated cells (*Figure 6-6E*). Moreover, analysis of surface-exposed proteins showed that activating PKA did not further alter the amount of α-ENaC in the membrane (*Figure 6-7C*), but did augment the surface abundance of β- and γ-ENaC (*Figure 6-7D, E*). Since these responses also differed from those seen in GC-deprived cells, this indicates that chronic (24 h) exposure to dexamethasone modifies the effects of cAMP agonists on the phosphorylation / expression of Nedd4-2 (*Figure 6-6*) and the surface expression of ENaC subunits (*Figure 6-7*).

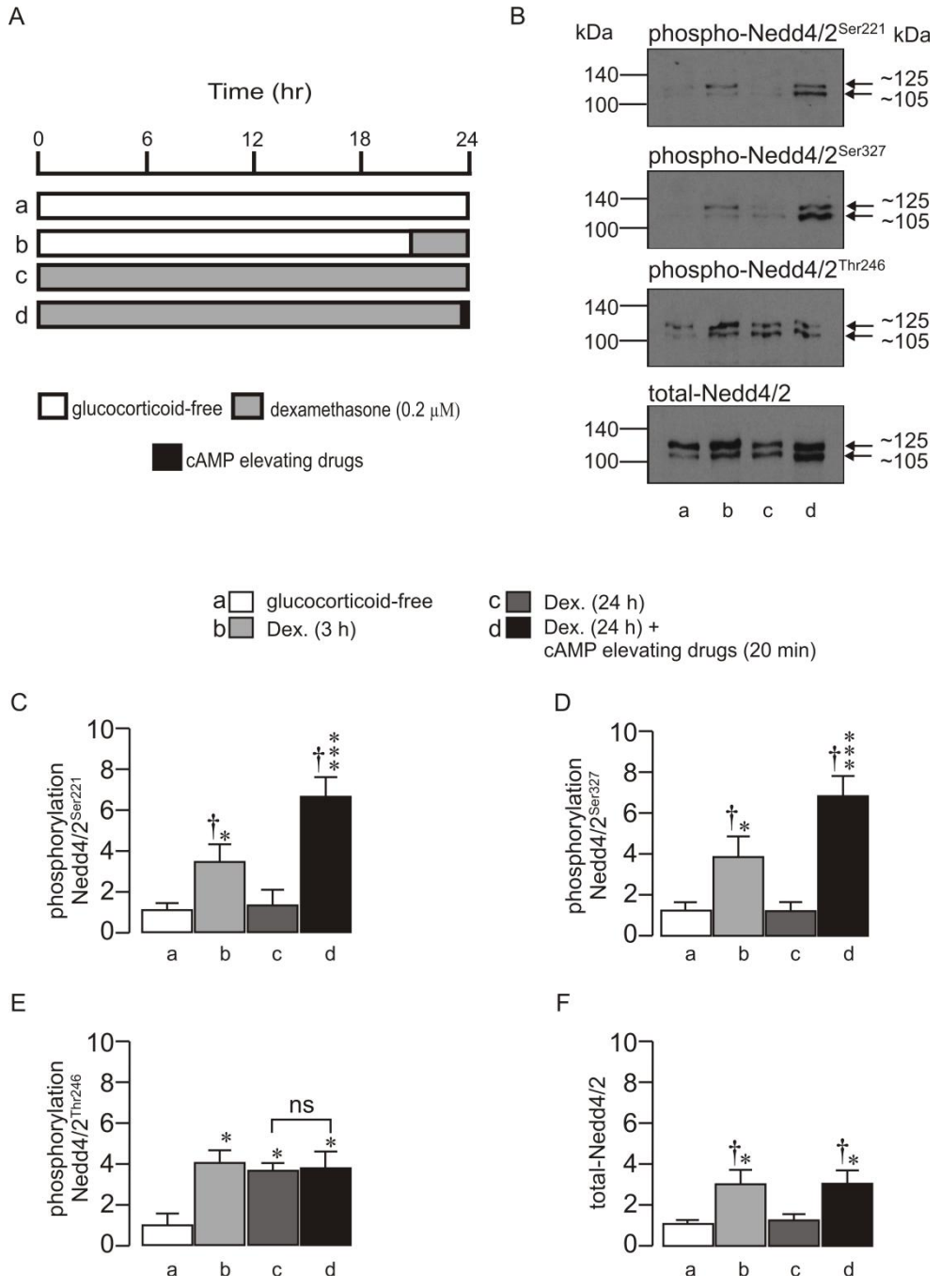


Figure 6-6: Effects of cAMP agonists on Nedd4-2 phosphorylated protein in unstimulated and dexamethasone-treated cells.

(A) Culture conditions prior to lysates. (B) Each blot illustrates the effects of cAMP agonists on the abundance of Nedd4-2 in dexamethasone-treated cells for 3 h and 24 h. Figures in (C), (D), (E) and (F) represent densitometry values for each subunit respectively. Pooled data from these values are shown as the mean ± SEM (n = 11). Asterisks denote statistically significant effects when compared to hormone deprived cells (control), *p<0.05, ***p<0.001. The dagger denotes a statistically significant difference between cAMP agonists and dexamethasone (24 h) treated cells; †p<0.05 (one-way ANOVA, Bonferroni *post-hoc* test).

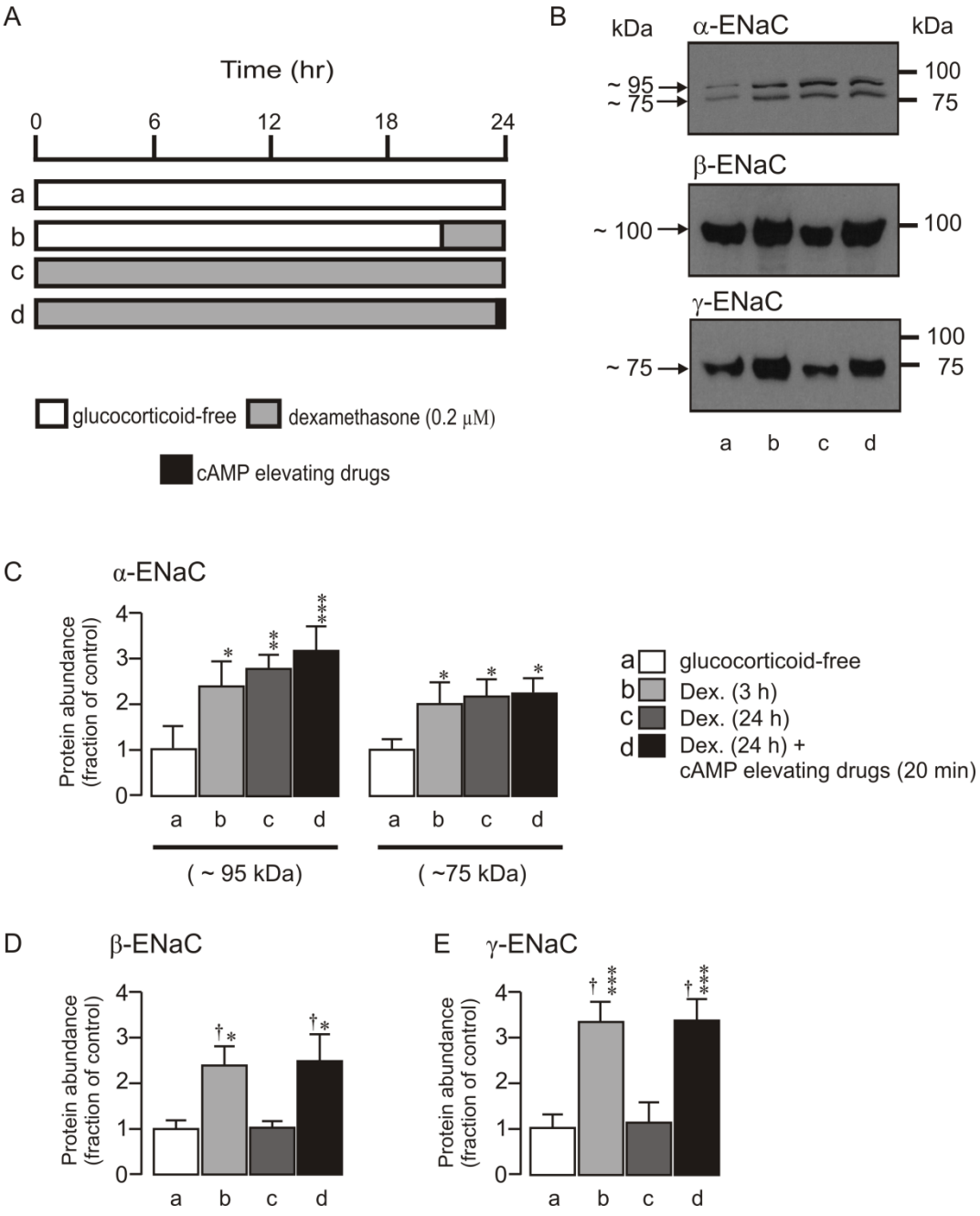


Figure 6-7: Effects of cAMP agonists on the abundance of cell surface ENaC in unstimulated and dexamethasone-treated cells.

(A) Culture conditions prior to cell extraction. Each blot illustrates the effects of cAMP agonists after incubation for 20 mins on the abundance of (B) ENaC at the cell surface (α-, β-, and γ-ENaC) in dexamethasone-treated cells for 3 h and 24 h. Figures in (C), (D) and (E) represent the densitometry values for each subunit respectively (α-ENaC, n = 11; β-ENaC, n = 7; γ-ENaC, n = 7) . Pooled data for these values are shown as the mean ± SEM. Asterisks denote statistically significant effects when compared to hormone deprived cells (control), *p<0.05, **p<0.01, ***p<0.001, The dagger denotes a statistically significant difference resulting from the effects of cAMP agonists compared to dexamethasone (24 h) treated cells; †p<0.05 difference (one-way ANOVA, Bonferroni *post-hoc* test).

6.3.5 Effects of GSK650394 on the responses to cAMP agonists in dexamethasone-treated cells on protein kinases

Exposing dexamethasone-treated (0.2 μM, 24 h) cells to GSK650394 (10 μM) for 3h reduced the abundance of $Thr^{346/356/366}$-phosphorylated NDRG1 without altering the overall NDRG1 expression level (*Figure 6-8D*), confirming that GSK650394 inactivates SGK1 (Sherk *et al.*, 2008). As anticipated, briefly (20 mins) exposing such cells to cAMP agonists evoked phosphorylation of CREB-Ser^{133} and, since this response was unaffected by GSK650394 (*Figure 6-8E*), it is clear that GSK650394 does not inhibit PKA. Moreover, we have recently shown that GSK650394 also has no effect upon phosphoinositide-3-kinase, phospholipid-dependent kinase 1, protein kinase B and the target of rapamycin signalling complex 2 (*Chapter 4*). This substance therefore provides a means by which to selectively inhibit SGK1 in human airway epithelial cells (Watt *et al.*, 2012).

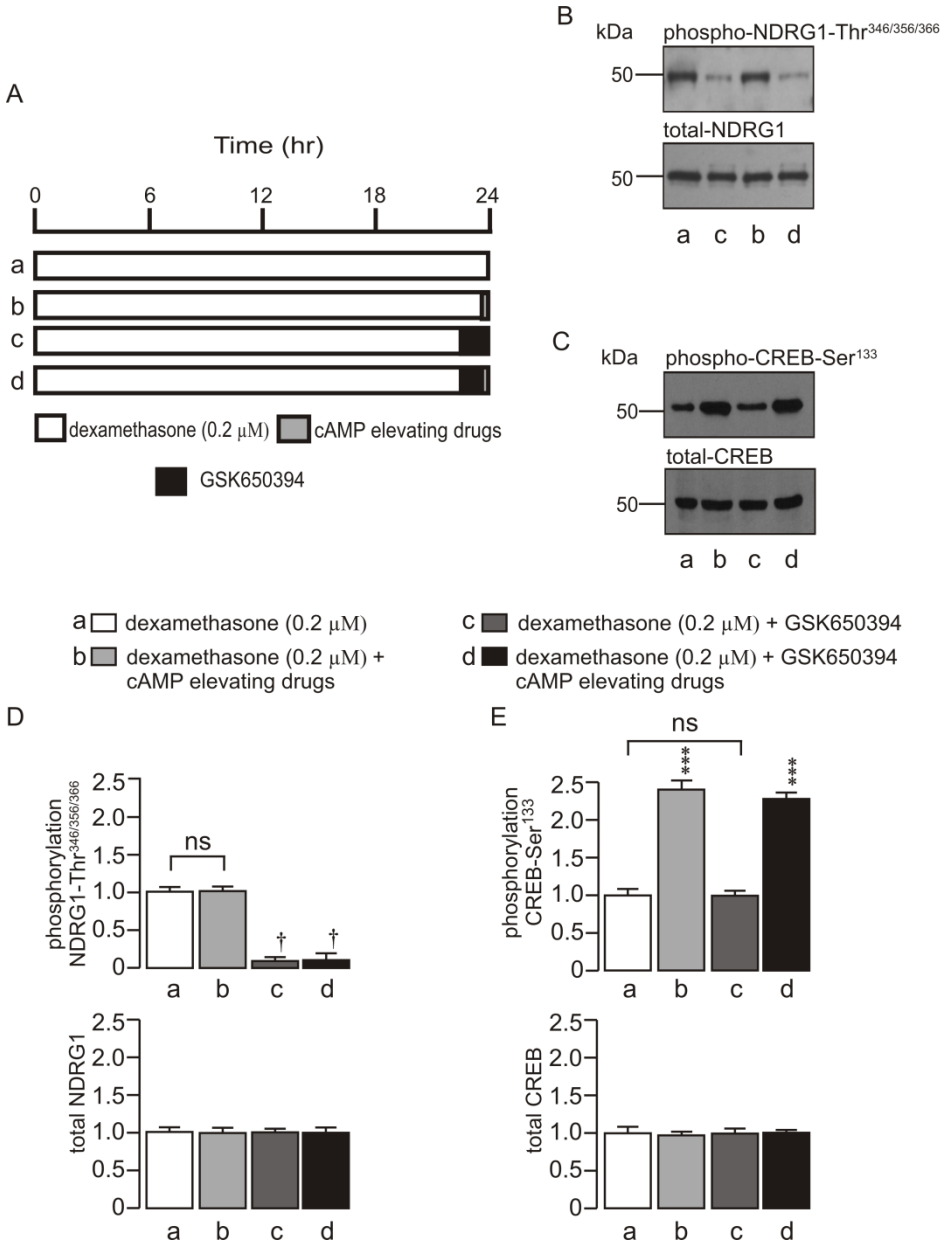


Figure 6-8: Effects of cAMP agonists on endogenous phosphorylation of CREB-Ser^{133} and NDRG1-Thr$^{346/356/366}$ in dexamethasone and GSK650394 treated H441 cells.

(A) H441 cells were exposed to dexamethasone (0.2 μM) for 24 h (control) and the SGK1 inhibitor GSK650394 to allow observation of the phosphorylation of CREB-Ser^{133} and NDRG1-Thr$^{346/356/366}$. A similar age-matched set of cells were pre-treated with dexamethasone for 24 h (control) and incubated with GSK650394 for the final 3 h and cAMP agonists for the final 20 min of the experiment. The upper blots show a Western analysis of the phosphorylated and total protein from (B) NDRG1-Thr$^{346/356/366}$ and (C) CREB-Ser^{133}. (D) and (E) show pooled data from 3 independent experiments represented as the mean ± SEM. Asterisks denote a statistically significant increase (***p<0.001). The dagger denotes a decreased effect compared to control cells, †p<0.01 (one-way ANOVA, Bonferroni *post-hoc*-test).

6.3.6 Effects of GSK650394 on the responses to cAMP agonists in dexamethasone-treated cells on Nedd4-2 and ENaC

Exposing dexamethasone-treated (0.2 μM, 24 h) cells to GSK650394 (10 μM, 3 h) reduced the abundance of the Ser^{221}- and Ser^{327}-phosphorylated forms of Nedd4-2 (*Figure 6-9 C,D*) without altering the overall Nedd4-2 expression level (*Figure 6-9 F*), a result which indicates that the basal phosphorylation of these residues must be maintained by SGK1. However, despite this clear finding, GSK650394 did not impair the phosphorylation of Nedd4-2-Thr^{246} (*Figure 6-9 E*) and the persistent phosphorylation of this residue in dexamethasone-treated (24 h) cells is therefore independent of SGK1. Exposing GSK650394-treated cells to cAMP agonists (20 mins) evoked clear increases in the abundance of Ser^{221}-phosphorylated, Ser^{327}-phosphorylated, Thr^{246}-phosphorylated and total forms of Nedd4-2 and it is therefore clear that the effects of PKA on the expression / phosphorylation of Nedd4-2 are independent of SGK1.

Parallel analysis of surface-exposed proteins showed that GSK650394 reduced the surface abundance of α- (*Figure 6-10C*), β- (*Figure 6-10D*), and γ-ENaC (*Figure 6-10E*), but, despite this clear finding, activating PKA under these conditions clearly increased the surface abundance of each subunit. Indeed, the responses to cAMP agonists seen in GSK650394-treated cells were virtually identical to the control and it is therefore clear the effects of cAMP agonists on the surface expression of ENaC subunits are also independent of SGK1.

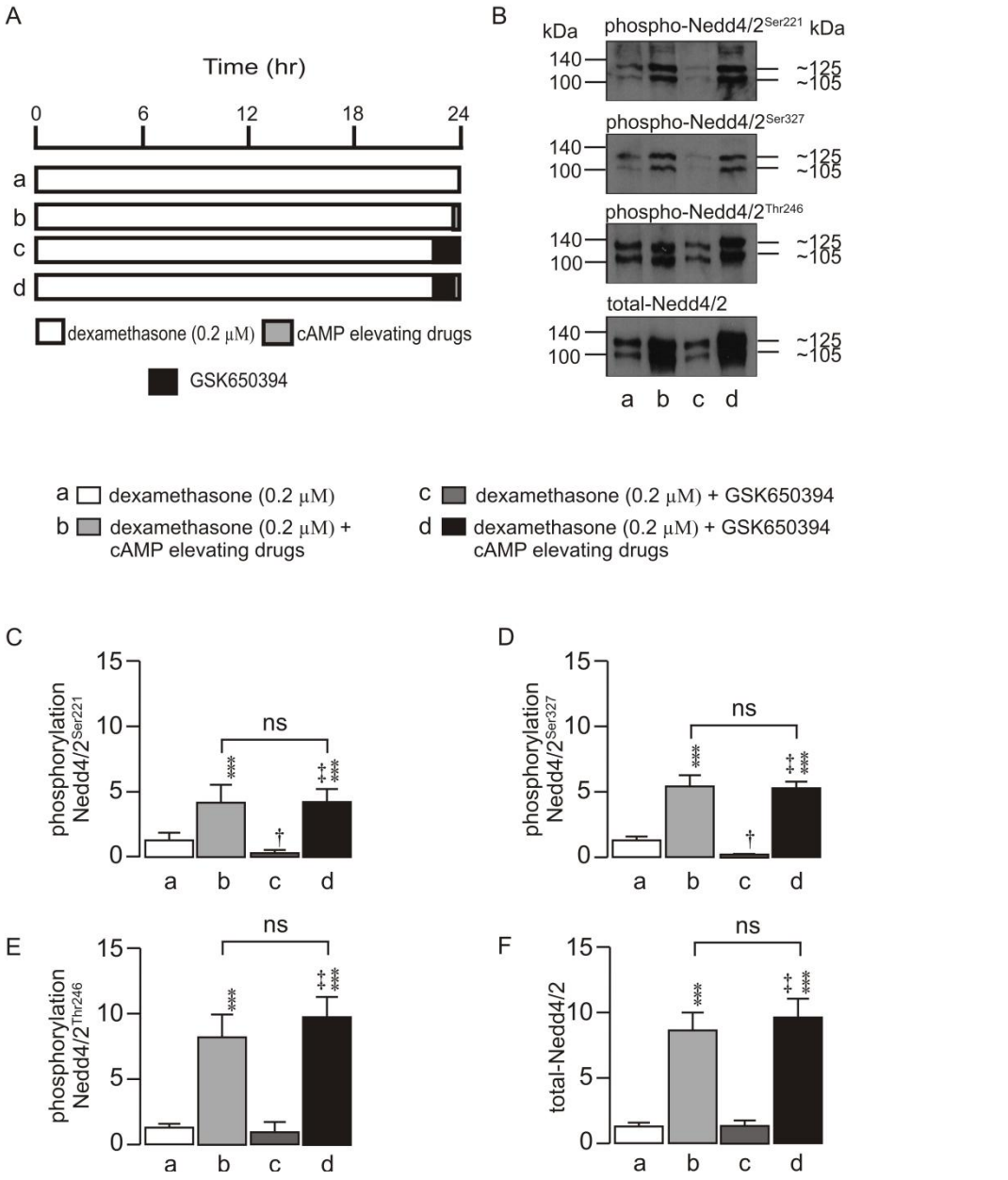


Figure 6-9: Effects of cAMP agonists on Nedd4-2 proteins in dexamethasone and GSK650394 treated cells.

(A) Cells were treated as indicated (B) Immunoblots of cells were treated with dexamethasone for 24 h and co-incubated with the SGK1 inhibitor compound GSK650394 for the final 3 h of the experiment. cAMP agonists were later added for the final 20 mins before cell extraction. The cell lysates were immuno-precipitated against a total Nedd4-2 antibody and processed through Western analysis. These proteins were probed for three residues of Nedd4-2 (Ser^{221}, Ser^{327}, and Thr^{246}) as well as total Nedd4-2. Each blot represents 3 independent experiments. The bar graphs in (C),(D), (E) and (F) show the mean ± SEM of the densitometry values in (A), (n = 4). Asterisks show a significant difference in the absence of cAMP agonists; ***p<0.001. The dagger shows a decreased effect compared to the control cells, †p<0.01. An increased effect of cAMP elevating drugs upon GSK650394 is denoted by ‡p<0.001 (one-way ANOVA, Bonferroni *post-hoc*-test).

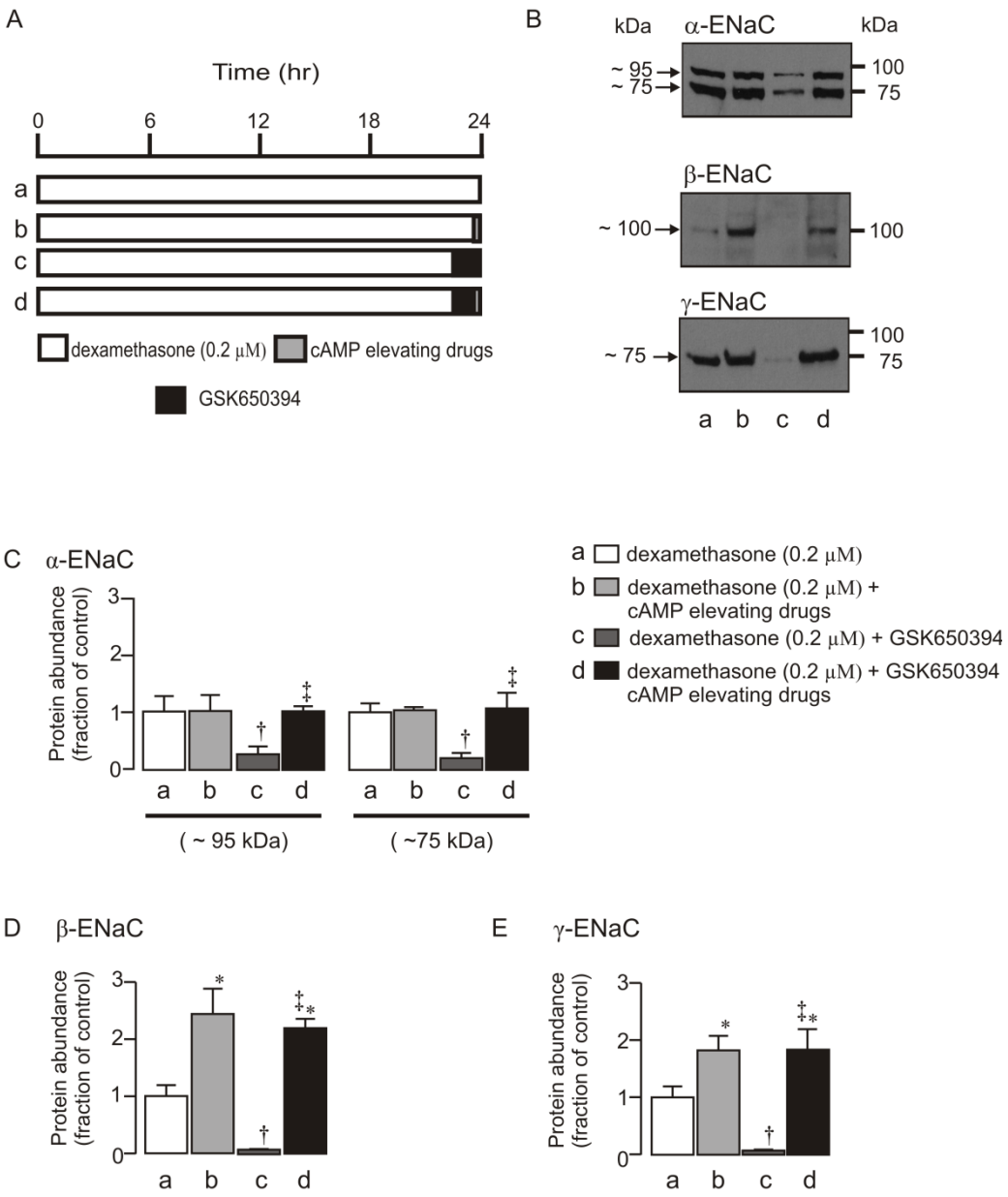


Figure 6-10: Effects of cAMP agonists on cell surface ENaC subunits in dexamethasone and GSK650394 treated cells.

(A) H441 cells were treated with dexamethasone for 24 h (control) and co-incubated with the SGK1 inhibitor compound; GSK650394 for the final 3 h of the experiment. cAMP agonists were added for the final 20 mins before cell extraction. The cell surface was labelled with biotin-bound streptavidin and processed using Western analysis. (B) These proteins were later probed for ENaC subunits (α-, β- and γ-). Each blot represents 4 independent experiments. Bar graphs in (C), (D) and (E) represent the densitometry values: mean ± SEM. Asterisks indicate a significantly increased effect compared to control cells, *p<0.05. The dagger shows a significantly decreased effect compared to control cells, †p<0.01. Double daggers indicate a sustainable significant effect of cAMP agonists on GSK650394, ‡p<0.01 (one-way ANOVA, Bonferroni *post-hoc*-test).

6.4 Discussion

ENaC expression can be regulated via phosphorylation and ubiquitination through different mechanisms. Among recent regulatory proteins found *in vitro*, the neural precursor cell-expressed, developmentally down-regulated isoform 4 protein (Nedd4-2) is known to ubiquitinate ENaC at the cell surface. This can be regulated by two different pathways: the serum GCs kinase 1 (SGK1) pathway (Debonneville *et al.*, 2001; Snyder *et al.*, 2002; Wiemuth *et al.*, 2010) that can be induced with GC hormones (eg: dexamethasone) and the cAMP dependent protein kinase A (PKA) pathway (Snyder, 2000; Snyder *et al.*, 2004a) by introducing cAMP agonists (eg: forskolin). Both pathways can phosphorylate multiple Thr-Ser residues of Nedd4-2 (Snyder, 2009) thereby reducing the affinity for ENaC and decreasing the inhibitory effect on ENaC at the cell surface. As a result, ENaC protein expression is augmented and there is an elevation in epithelial Na^+ absorption. This suggests that Nedd4-2 plays a central role in regulating Na^+ transport in these pathways (Snyder *et al.*, 2004a). This study also found that Nedd4-2 can be a substrate for phosphorylation both *in vitro* and *in vivo*.

6.4.1 Cyclic AMP agonists activate PKA and SGK1

Exposing GC-deprived cells to cAMP agonists increased the activity of PKA and SGK1. A similar response has been demonstrated by previous studies which reported that PKA activated SGK1 by phosphorylating SGK1-Thr^{389} (Morris *et al.*, 2002; Chandran *et al.*, 2011). Moreover, cAMP-coupled agonists have also been shown to increase the abundance of SGK1 mRNA and protein in absorptive epithelial cells (Vasquez *et al.*, 2008) providing evidence for cross talk between the PKA-dependent and SGK1-dependent pathways. Indeed, it has been suggested that that cAMP / PKA-coupled agonists might control Na^+ transport via a mechanism dependent on SGK1 (Gonzalez *et al.*, 2000; Perrotti *et al.*, 2001; Thomas *et al.*, 2004; Vasquez *et al.*, 2008). However, whilst the activation of PKA

described here was complete within ~20 mins, SGK1 activation involved a latency of 1 – 2 h, and physiological studies of absorptive epithelia have shown that cAMP-coupled agonists can induce Na^+ transport within 5 – 10 min (Morris *et al.*, 2002; Lazrak *et al.*, 2003; Thomas *et al.*, 2004; Ramminger *et al.*, 2004; Clunes *et al.*, 2004; Mansley & Wilson, 2010a). Although SGK1 could contribute to the maintenance of stimulation, PKA-induced activation of SGK1 cannot account for the rapid responses to cAMP-coupled agonists as seen in human airway epithelial cells (Lazrak *et al.*, 2003; Thomas *et al.*, 2004; Ramminger *et al.*, 2004; Clunes *et al.*, 2004).

6.4.2 Cyclic AMP agonists alter the expression / phosphorylation of Nedd4-2

Exposing GC-deprived cells to cAMP agonists increased the abundance of the Ser^{221}- and Ser^{327}-phosphorylated forms of Nedd4-2 and these responses initially followed a time course similar to the activation of PKA. Whilst these data suggest that PKA might phosphorylate Nedd4-2 at Ser^{221} and -Ser^{327}, the cAMP agonists also caused clear increases in the overall abundance of Nedd4-2 and, initially, this response followed a time course similar to that seen with the $Ser^{221/327}$-phosphorylated protein (Snyder *et al.*, 2004a; Chandran *et al.*, 2011). The apparent phosphorylation of these residues may thus be due, at least in part, to an increase in overall abundance. It is therefore interesting that studies of heterologously expressed *Xenopus* Nedd4-2 have shown that the degradation of Nedd4-2 is blocked by the phosphorylation of a residue equivalent to human Nedd4-2-Ser^{327}, and stimuli that promote the phosphorylation of this residue can thus increase the overall abundance and prevent its degradation (Chandran *et al.*, 2011). Indeed, it has been suggested that such control over the abundance of Nedd4-2 might contribute to the hormonal control of Na^+ absorption (Chandran *et al.*, 2011). Whilst the present data are consistent with this hypothesis, studies of murine cortical collecting duct cells suggest that the aldosterone-induced phosphorylation of a residue equivalent to human Nedd4-2-Ser^{327}

has no effect on the overall abundance of this protein (Flores *et al.*, 2005). Moreover, although the activation of PKA was well maintained, prolonged exposure to cAMP agonists led to a reduction in the overall abundance of Nedd4-2 and this protein thus became virtually undetectable after 12 – 24 h. The effects of cAMP agonists on Nedd4-2 expression in human airway epithelial cells therefore appears to be biphasic, however the reason for this is unknown. More detailed studies of endogenous Nedd4-2 using fully quantitative methods are therefore needed to define the changes to the expression / phosphorylation of this protein that accompany regulated Na^+ transport.

6.4.3 PKA and SGK1 have different effects on Nedd4-2 and ENaC in GC-deprived cells

Although the cAMP agonists did increase the abundance of Thr^{246}-phosphorylated Nedd4-2, this response developed much more slowly that the effects on the $Ser^{221/327}$-phosphorylated forms of this protein and therefore coincide with the delayed activation of SGK1. This observation raises the possibility that Nedd4-2-Thr^{246} might be a substrate for SGK1 and not PKA. Further evidence for this came from studies of cells that had been briefly exposed to dexamethasone for 3 h in order to activate SGK1 independently of PKA. This stimulus increased the abundance of the Ser^{221}-phosphorylated, Ser^{327}-phosphorylated and total Nedd4-2, and these responses resembled those seen in cAMP-stimulated cells. However, dexamethasone also evoked a clear increase in the abundance of Thr^{246}-phosphorylated Nedd4-2, and this additional effect was confirmed by a separate series of experiments that used a strictly paired experimental design to compare the effects PKA and SGK1 directly. Work undertaken by Snyder *et al.* is relevant in this context since these authors showed that Nedd4-2 could be inactivated by PKA or SGK1 (Snyder *et al.*, 2002; Snyder *et al.*, 2004a). However, since PKA acted by phosphorylating the protein at Ser^{327} and Ser^{221}, but not at Thr^{246}, the authors then proposed that the inhibitory action of SGK1 was due to the

phosphorylation of Ser^{327} and Thr^{246}, but not Ser^{221} (Snyder *et al.*, 2002; Snyder *et al.*, 2004a). Moreover, recent studies of heterologously expressed *Xenopus* Nedd4-2 show that the retrieval of ENaC subunits from the plasma membrane is dependent on the phosphorylation of Nedd4-2 at sites equivalent to Nedd4-2-Thr^{246} and Nedd4-2-Ser^{221} (Chandran *et al.*, 2011). Similarly, studies using mass spectroscopy have suggested that aldosterone-induced Na^+ transport in mouse cortical collecting duct cells involves the phosphorylation of Nedd4-2 at a site equivalent to Thr^{246} (Chandran *et al.*, 2011).

A considerable body of work now suggests that the regulated phosphorylation of Nedd4-2-Thr^{246} is central to the control of Na^+-transport (Snyder *et al.*, 2004a; Chandran *et al.*, 2011). These data suggest that the phosphorylation of Nedd4-2-$Ser^{221/327}$ might not provide a stimulus that can sufficiently suppress internalisation / degradation of ENaC. It is therefore interesting that analysis of surface-exposed proteins showed that cAMP agonists increased the amount of α-ENaC in the membrane but had no effect upon the surface abundance of β- or γ-ENaC. The fact that the cAMP agonists could control the surface abundance of α-ENaC was surprising and we have no clear explanation for this effect, although signalling via cAMP / PKA can increase the overall expression of α-ENaC by inducing gene expression. However, the most important result to emerge from the experiments presented here was that selective activation of PKA in GC-deprived cells did not provide a stimulus that was sufficient to evoke a coordinated increase the surface expression of α-, β- and γ-ENaC. Parallel studies of dexamethasone-stimulated (3 h) cells, on the other hand, confirmed that this synthetic GC does increase the surface abundance of all three subunits, and these data therefore support the view that phosphorylation of Nedd4-2-Thr^{246} is critical for controlling the abundance of ENaC subunits at the membrane.

6.4.4 Prolonged (24h) exposure to dexamethasone evokes Nedd4-2-Thr^{246} phosphorylation

The fact that the cAMP agonists were unable to regulate the surface abundance of all three ENaC subunits was surprising since cAMP-agonists do stimulate Na^+ transport in H441 cells (Lazrak *et al.*, 2003; Thomas *et al.*, 2004; Ramminger *et al.*, 2004; Clunes *et al.*, 2004; Vasquez *et al.*, 2008) and this response is almost invariably attributed to changes in the surface abundance of ENaC subunits (Morris *et al.*, 2002). However, these earlier studies were undertaken using cells that had been exposed to GCs and / or serum before being used in experiments (Lazrak *et al.*, 2003; Thomas *et al.*, 2004; Ramminger *et al.*, 2004; Clunes *et al.*, 2004), whereas the present experiments were undertaken using GC-deprived cells. Further experiments therefore explored the effects of stimulating cells with dexamethasone for ~24 h.

Our initial analysis of the extracted proteins confirmed that the GC-induced activation of SGK1 could not be sustained for 24 h (*Chapter 3*; see also Watt *et al.*, 2012; Inglis *et al.*, 2009) and, although the surface abundance of α-ENaC was elevated, the amounts of β- and γ-ENaC present in the membrane were similar to the levels measured in GC-deprived cells. Although ENaC-dependent Na^+ currents have been recorded from such dexamethasone-treated cells (Lazrak *et al.*, 2003; Ramminger *et al.*, 2004; Clunes *et al.*, 2004), the data presented here confirms that these currents cannot be attributed to a coordinated increase in the surface abundance of α-, β- and γ-ENaC (Watt *et al.*, 2012). Moreover, the effects of dexamethasone (3 h) on the abundance of Ser$^{221/327}$-phosphorylated and total Nedd4-2 did not persist for ~24 h, a response that correlates well with changes to the activity of SGK1. Prolonged (24 h) exposure to dexamethasone did, however, cause a persistent increase in the abundance of the Thr^{246}-phosphorylated Nedd4-2 and, since this was not associated with

any change in overall expression, it is now clear that chronic exposure to dexamethasone evokes sustained phosphorylation of Nedd4-2 at Thr^{246}.

6.4.5 Prolonged (24h) exposure to dexamethasone modifies the response to cAMP agonists

Exposing dexamethasone-treated (24 h) cells to cAMP agonists increased the abundance of the Ser^{221}-phosphorylated, Ser^{327}-phosphorylated and total forms of Nedd4-2 but, in contrast to the situation seen in GC-deprived cells, these responses were accompanied by an increase in the abundance of the Thr^{246}-phosphorylated Nedd4-2. Moreover, the cAMP agonists also increased the surface abundance of β- and γ-ENaC in such cells, whilst functional studies showed that chronic (24 h) dexamethasone stimulation also enhanced the electrometric response to cAMP agonists. Prolonged (24 h) exposure to GC hormones therefore enhances the effects of cAMP, so it is interesting that earlier studies of pulmonary epithelia showed that cAMP-coupled agonists can only evoke increased Na^+ transport in cells that have been exposed to GC / thyroid hormones (Barker *et al.*, 1991; Ramminger *et al.*, 2002; Olver *et al.*, 2004). These hormones thus exert a permissive action that is an important aspect of lung development (Olver *et al.*, 1986; Barker *et al.*, 1991; Ramminger *et al.*, 2002; Olver *et al.*, 2004).

Whilst our data (see also Snyder *et al.*, 2002; Snyder *et al.*, 2004a; Chandran *et al.*, 2011) suggests that phosphorylation of Nedd4-2-Thr^{246} is needed to control the surface expression of ENaC subunits, this residue does not seem to be a PKA substrate and this may well explain why cAMP agonists cannot evoke coordinated increases in the surface abundance of the ENaC subunits in GC deprived cells. Moreover, by demonstrating that prolonged (24 h) exposure to GCs leads to the sustained phosphorylation of Nedd4-2-Thr^{246}, we have identified a mechanism that may allow GCs to facilitate the responses to cAMP-coupled

agonists. Since many effects of GC hormones are mediated via SGK1, we also explored the effects resulting from exposure of dexamethasone-treated (24 h) cells to GSK650394 in order to inactivate this kinase. GSK650394 reduced the abundance of the Ser^{221}- and Ser^{327}-phosphorylated forms of Nedd4-2 indicating that the phosphorylation of these residues in dexamethasone-treated cells is dependent on SGK1. However, GSK650394 did not suppress the phosphorylation of Nedd4-2-Thr^{246}, and the sustained phosphorylation of this residue must therefore depend on a kinase other than SGK1. Identifying the mechanisms that allow GCs to maintain the phosphorylation of Nedd4-2-Thr^{246} may thus clarify the way in which pulmonary Na^+ transport processes are initiated, maintained and regulated and this, in turn, may facilitate the development of novel therapeutic strategies to treat diseases that involve disrupted pulmonary Na^+ transport.

GSK650394 also reduced the surface abundance of α-, β- and γ-ENaC in dexamethasone-treated (24 h) cells and these data are consistent with the view that the SGK1-dependent phosphorylation of Nedd4-2 is a major determinant of the surface abundance of the ENaC subunits (Snyder *et al.*, 2002; Flores *et al.*, 2005; Debonneville *et al.*, 2001; Lang *et al.*, 2006; Snyder, 2005). However, GSK650394 did not block the effects of cAMP agonists upon the phosphorylation / expression of Nedd4-2 or the surface expression of the ENaC subunits. Moreover, whilst GSK650394 did inhibit the rate of electrogenic Na^+ transport, it did not suppress the electrometric response to cAMP agonists and, similarly, we have shown that cAMP-coupled agonists can regulate Na^+ transport via an SGK1-independent mechanism in epithelial cells from the airway and distal nephron (Mansley & Wilson, 2010a). It is therefore interesting that Snyder *et al.* have proposed that PKA controls Na^+ transport by directly phosphorylating Nedd4-2, a hypothesis that predicts that the responses to cAMP-coupled agonists should occur independent of SGK1 (Snyder *et al.*, 2004a).

7

Conclusions and future work

7.1 Conclusions

To date, the endogenous expression of ENaC proteins in human airway H441 cells has not been fully explored due to the complexity of protein detection using Western analysis. Previously, most studies were conducted by either over expressing or knocking out ENaC. However, in this thesis ENaC activity was studied using a cell surface biotin-labelled protein technique, thus revealing the association between ENaC protein expression and Na^+ conductance.

The treatment of airway cells with dexamethasone improves the prognosis of patients suffering from respiratory distress syndrome and pulmonary oedema. This effect has also been demonstrated experimentally in an H441 lung cell model that showed an increased Na^+ current (Ramminger *et al.*, 2004; Clunes *et al.*, 2004; Brown *et al.*, 2008; Inglis *et al.*, 2009; Watt *et al.*, 2012). Dexamethasone appears to activate the SGK1 protein indicator, NDRG1-$Thr^{346/356/366}$, resulting in an increase in expression of α-ENaC in both pools of proteins, which may then potentiate Na^+ absorption. The expression of β-ENaC is only

found in the total lysate during a longer period of activation as a result of a transient effect, indicating that β-ENaC is only involved in the internalisation of the channel. Since γ-ENaC is involved in both the brief and prolonged activation at the cell surface, this suggests that it assists β–ENaC in enhancing the internalisation of the channel and further elevates the functional expression of α-ENaC at the cell surface.

The mechanism that lies downstream of dexamethasone is thought to be dependent on PI3-kinase – TORC2 – SGK1 – Nedd4-2 (Blazer-Yost *et al.*, 1998; Record *et al.*, 1998; Alvarez de la Rosa *et al.*, 1999; Paunescu *et al.*, 2000; Debonneville *et al.*, 2001; Alvarez de la Rosa & Canessa, 2003; Blazer-Yost *et al.*, 2003; Inglis *et al.*, 2009; Boase *et al.*, 2011), leading to the increased expression of ENaC subunits at the cell surface. SGK1 is thought to play a role in coordinating the increase in ENaC expression at the cell surface which must be mediated through PI3-kinase. The introduction of compounds such as PI-103, TORIN1 and GSK650394 inhibit this mechanism, but such effects only cause a modest decrease in the number of ENaC subunits. However, brief treatment with dexamethasone in the presence of these inhibitors abolished the β- and γ- subunits, and slightly reduced the amount of α-ENaC, suggesting that SGK1 plays a role in controlling ENaC protein at the cell surface. Since there were only minor changes in the level of α-ENaC in response to these inhibitors, this suggests that there is another mechanism that controls the expression of this subunit at the surface membrane.

Since ENaC proteins are maintained by the SGK1 pathway downstream of PI3K, we predict that this mechanism must also influence the expression of the ubiquitin protein ligase Nedd4-2. The data in *Chapter 5* showed that the expression of Nedd4-2 is transient, and corresponds to the activation of SGK1 to further inhibit the interaction with the PY motif of ENaC (Debonneville *et al.*, 2001; Snyder *et al.*, 2002). As a result of this, there was a

significant elevation in expression of ENaC subunits at the cell surface. This finding is in contrast to a similar study which used cells that over-expressed Nedd4-2 (Debonneville *et al.*, 2001; Snyder *et al.*, 2002; Snyder *et al.*, 2004a); the endogenous processed cells used in this study are thought to better reflect a native system. All three residues of Nedd4-2 (Ser^{221}, Thr^{246} and Ser^{327}) were elevated prior to brief exposure to dexamethasone (3 h) to reflect the increment of whole expression of Nedd4-2 total protein. Inhibition with GSK650394 reduced SGK1 activity and abolished the expression of Nedd4-2, showing that Nedd4-2 activation is dependent on SGK1. The data presented in this thesis revealed that the expression of GC-induced ENaC subunits at the cell surface is dependent on PI3-kinase – TORC2 – SGK1 – Nedd4-2. Whilst the data supports this hypothesis, it does not clarify the magnitude of the Na^+ current (Watt, 2011). However, this can be explained by the fact that SGK1 can also augment the membrane Na^+ current without recruitment of all channels to the membrane, though it is dependent on the SGK1-consensus motif in the C-terminal region of α-ENaC (Diakov & Korbmacher, 2004). However, this pathway still requires further study.

Though Nedd4-2 proteins are maintained through the SGK1 pathway, other studies have suggested that ubiquitin protein ligase can be induced by another signalling pathway, such as Protein Kinase A (PKA) through cyclic AMP (for example: Snyder *et al.*, 2004a). PKA activating agonists containing forksolin with IBMX and bucladesine activate amiloride sensitive GC-induced ENaC activity (Clunes *et al.*, 2004). The data in this thesis has shown that a cocktail of cAMP agonists can activate ENaC surface expression through PKA. Increased expression of Nedd4-2 elevates ENaC subunits at the cell surface, which is PKA dependent. The brief stimulation by this cocktail is independent of the SGK1 pathway, as PKA is shown to be active in the presence of the SGK1 inhibitor, GSK650394. However, when the cocktail was added to dexamethasone-treated cells, the PKA-activating agonists

modified the response to dexamethasone, resulting in a further increase in both Nedd4-2 and ENaC. This increment is parallel to the activation of Nedd4-2- Thr^{246} but not other phopshorylation sites suggesting the important role of Thr^{246} as a key player to regulate ENaC at the cell surface.

Finally, the conclusions taken from this thesis are summarised below:

- Glucocorticoids induce a transient increase in SGK1 activity in H441 cells.

- Brief activation of SGK1 increases the coordinated expression of α-, β-, γ- ENaC at the surface membrane of H441 cells.

- Prolonged activation of glucocorticoids (24 h) results in sustained expression of α-ENaC and an increase in γ-ENaC at the membrane, resulting in the induction of amiloride-sensitive ENaC conductance (Watt, 2011).

- The mechanism by which glucocorticoids regulate ENaC cell surface expression in H441 cells involves the PI3K-TORC2-SGK1 pathway.

- The absence of PI3K/SGK1 leads to the withdrawal of ENaC subunits (especially β- and γ-ENaC) from the membrane, suggesting an important role for PI3K/SGK1 in glucocorticoid-treated H441 cells.

- Sustained expression of α-ENaC in the absence of PI3K/SGK1 indicates that the α-subunit can be activated independently of the above mentioned mechanism.

- The same mechanism that glucocorticoids use to regulate ENaC cell surface expression in H441 cells is used for regulating the expression of the ubiquitin ligase protein, Nedd4-2 (Ser^{221}, Ser^{327}, Thr^{246}). This is dependent on the PI3K-TORC2-SGK1 pathway.

- Cyclic AMP agonists can regulate ENaC surface expression in H441 cells through activation of the PKA pathway.

- ENaC surface expression can be independently regulated by the PKA and SGK1 pathways in H441 cells, but when cAMP agonists are added to dexamethasone-treated cells this results in the modification of ENaC expression, resulting in an increased natriferic response (Clunes *et al.*, 2004).

- This modified response results from the phosphorylation of the Thr^{246} residue of Nedd4-2, suggesting a convergence role for Nedd4-2 between PKA and SGK1 during the regulation of ENaC at the surface membrane of H441 cells.

7.2 Future work

The data from this thesis confirms that the H441 human airway cell line can be used as a model to investigate ENaC subunit expression and the hormonal control of the SGK1 and PKA pathways. Analysis in vivo could be performed using primary cell culture and lung slices or human nasal epithelia, obtained from nasal scrapings. This study only focused on the role of the SGK1 and PKA pathways, however, there are many regulators of ENaC that should be considered for future experiments. Other regulators might involve GILZ, which may be involved in the proteolytic cleavage and independent activation of α-ENaC. Studying other potential regulators will help to explain any compensatory effects seen *in vivo*.

In addition to studies on living cells, it would be interesting to investigate the role of other hormones on different cell lines, as there may be an alternative model to explain the role of SGK1 and PKA or implicate other pathways. We could investigate the effects of the hormones on human bronchiolar epithelial cells or cultures of isolated ATI and ATII cells from lung slice preparations. These studies would help to clarify the role of each pathway (in this case: SGK1 and PKA) in maintaining ENaC expression and controlling Na^+ transport. Understanding how these pathways work in this context is crucial for the development of better treatments. For example, a drug that successfully inhibits SGK1 could be developed to treat Liddle's syndrome, a condition that is underpinned by hyperactivation of ENaC.

Since ENaC expression at the cell surface can be evoked by both dexamethasone and cAMP agonists, it is thought to be dependent on the SGK1 and PKA pathways via the activation of Nedd4-2. Both β- and γ-ENaC are involved in internalisation as they bind to Nedd4-2 at the

PY motif, therefore it would be interesting to coimmunoprecipitate the cellular extracts with both total Nedd4-2 and each subunit of ENaC to see if there is a specific interaction.

To date, a number of studies have proposed that 14-3-3 proteins mediate the interaction between Nedd4-2 and ENaC at the surface membrane by direct interaction with a phosphorylated form of human Nedd4-2 (Ichimura *et al.,* 2005; Nagaki *et al.,* 2006). Therefore, it would be useful to determine the final effector of the mechanism that completes ENaC expression at the cell surface. The interaction between Nedd4-2 and 14-3-3 proteins can be observed by processing the cell lysates through an immuno-precipitation procedure against total Nedd4-2, then probing for 14-3-3 protein. The combined expression of both Nedd4-2 and 14-3-3 is also indicated by the effect on ENaC activity. Since there is no specific inhibitor known to affect the functional response of these proteins, a knock-down of Nedd4-2 (Kimura *et al.*, 2011) and/or 14-3-3 would provide an insight into its role in hormonally regulated ENaC activity.

Prolonged treatment of cAMP modifies the effect of SGK1 on ENaC at the cell surface, suggesting an association between the PKA and SGK1 pathways. Data from this thesis revealed that the expression of ENaC can be independent of either pathway individually, suggesting that there may be a mechanism that lets them work synergistically. To confirm this, we would need to test the effects of prolonged exposure to a number of stimulators/inhibitors of both pathways on ENaC/Nedd4-2/14-3-3 expression.

SGK1 certainly serves as an important regulator for maintaining ENaC at the cell surface, but there is another novel mechanism that is known to regulate a similar pathway. WNK (with no lysine (K)) protein kinase affects ion balance and mutations at the active site lysine cause a heritable form of ion imbalance, culminating in hypertension. There are a number of

ways that this family of proteins could regulate the pathway. A recent study found strong interactions between four WNK proteins and SGK1, especially WNK1 (Heise *et al.*, 2010). Moreover, WNK1 is involved in Nedd4-2 binding, thus it can inhibit the Nedd4-2 – ENaC interaction at the cell surface, therefore promoting channel expression. Since this mechanism is similar to the activation of SGK1, it may be worthy of further study and could contribute to a more comprehensive understanding of the mechanisms that underpin ENaC expression.

References

ABRIEL, H., LOFFING, J., REBHUN, J. F., PRATT, J. H., SCHILD, L., HORISBERGER, J. D., ROTIN, D. & STAUB, O. (1999). Defective regulation of the epithelial Na^+ channel by Nedd4 in Liddle's syndrome. *J Clin Invest.,* **103:** 667-73.

ADAMS, F.H., MOSS, A.J., FAGAN, L., 1963. The tracheal fluid in the fetal lamb. *Biol. Neonate* 5, 151–158.

ADAMS, C. M., SNYDER, P. M. & WELSH, M. J. (1997). Interactions between subunits of the human epithelial sodium channel. *J Biol Chem.,* **272:** 27295-300.

ADAMSON TM, BOYD RDH, PLATT HS & STRANG LB. (1969). Composition of alveolar liquid in the foetal lamb, pp. 159. Physiological Soc.

ADEBAMIRO, A., CHENG, Y., JOHNSON, J. P. & BRIDGES, R. J. (2005). Endogenous protease activation of ENaC: Effect of serine protease inhibition on ENaC single channel properties. *J Gen Physiol.,* **126:** 339-52.

AHERNE W, DAWKINS M.J. (1964). The removal of fluid from the pulmonary airways after birth in the rabbit and the effect on this of prematurity and pre-natal hypoxia. *Biol Neonat.,* **7:** 214-29.

AHN, Y. J., BROOKER, D. R., KOSARI, F., HARTE, B. J., LI, J., MACKLER, S. A. & KLEYMAN, T. R. (1999). Cloning and functional expression of the mouse epithelial sodium channel. *Am J Physiol,* **277,** 121-9.

ALBERT, A. P., WOOLLHEAD, A. M., MACE, O. J. & BAINES, D. L. (2008). AICAR decreases the activity of two distinct amiloride-sensitive Na^+-permeable channels in H441 human lung epithelial cell monolayers. *Am J Physiol-Lung Cell Mol Physiol,* **295**: 837-48.

ALESSI, D. R. & COHEN, P. (1998). Mechanism of activation and function of protein kinase B. *Curr Opin Genet Dev.,* **8:** 55-62.

ALTHAUS M, PICHL A, CLAUSS WG, SEEGER W, FRONIUS M, MORTY RE. (2010). Nitric Oxide Inhibits Highly Selective Sodium Channels and the Na^+/K^+-ATPase in H441 Cells. *Am. J. Respir. Cell Mol. Biol.* 4,53-65.

ALVAREZ DE LA ROSA, D. & CANESSA, C. M. (2003). Role of SGK in hormonal regulation of epithelial sodium channel in A6 cells. *Am J Physiol Cell Physiol,* **284:** 404-14.

ALVAREZ DE LA ROSA, D., ZHANG, P., NARAY-FEJES-TOTH, A., FEJES-TOTH, G. & CANESSA, C. M. (1999). The serum and glucocorticoid kinase sgk increases the abundance of epithelial sodium channels in the plasma membrane of Xenopus oocytes. *J Biol Chem,* **274:** 37834-9.

ANANTHARAM, A. & PALMER, L. (2007). Determination of epithelial Na^+ channel subunit stoichiometry from single-channel conductances. *J Gen Physiol.***, 130**:55-70.

ANDERSON, M., P., SHEPPARD, D., N., BERGER, H., A. & WELSH, M., J. (1992). Chloride channels in the apical membrane of normal and cystic fibrosis airway and intestinal epithelia. *Am J Physiol Lung Cell Mol Physiol*, **263:** 1-14.

ANDJELKOVIC M, ALESSI DR, MEIER R, FERNANDEZ A, LAMB NJC, FRECH M, CRON P, COHEN P, LUCOCQ JM & HEMMINGS BA. (1997). Role of translocation in the activation and function of protein kinase B, pp. 31515. ASBMB.

ARAKI, I., DU, S., KAMIYAMA, M., MIKAMI, Y., MATSUSHITA, K., KOMURO, M., FURUYA, Y. & TAKEDA, M. (2004). Overexpression of epithelial sodium channels in epithelium of human urinary bladder with outlet obstruction. *Urol.,* **64:** 1255-60.

ASHER, C., SINHA, I. & GARTY, H. (2003). Characterization of the interactions between Nedd4-2, ENaC, and sgk-1 using surface plasmon resonance. *Biochim Biophys Acta,* **1612:** 59-64.

AWAYDA, M. S., BENGRINE, A., TOBEY, N. A., STOCKAND, J. D. & ORLANDO, R. C. (2004). Nonselective cation transport in native esophageal epithelia. *Am J Physiol Cell Physiol.,* **287:** 395-402.

AWAYDA, M. S., ISMAILOV, I. I., BERDIEV, B. K., FULLER, C. M. & BENOS, D. J. (1996). Protein kinase regulation of a cloned epithelial Na^+ channel. *J Gen Physiol,* **108:** 49-65.

AWAYDA, M. S., TOUSSON, A. & BENOS, D. J. (1997). Regulation of a cloned epithelial Na^+ channel by its beta- and gamma-subunits. *Am J Physiol.,* **273:** 1889-99.

BAIN, J., PLATER, L., ELLIOTT, M., SHPIRO, N., HASTIE, C. J., MCLAUCHLAN, H., KLEVERNIC, I., ARTHUR, J. S. C., ALESSI, D. R. & COHEN, P. (2007). The selectivity of protein kinase inhibitors: a further update. *Biochem J,* **408:** 297-315.

BAINES, D., L., FOLKESSON, H., G., NORLIN, A., BINGLE, C. D., YUAN, H. T. & OLVER, R. E. (2000). The influence of mode of delivery, hormonal status and postnatal O_2 environment on epithelium sodium channel (ENaC) expression in perinatal guinea-pig lung. *J Physiol.* **522:** 147-57.

BARKER, P., M., BROWN, M., J., RAMSDEN, C., A., STRANG L. B. & WALTERS D.V., (1988). The effect of thyroidectomy in the fetal sheep on lung liquid reabsoprtion induced by adrenaline or cyclic AMP. *J Physiol.* **407:** 373-83.

BARKER P.M., GATZY J.T. (1993) Effect of gas composition on liquid secretion by explants of distal lung of fetal rat in submersion culture. *Am J Physiol Lung Cell Mol Physiol* 265: L512–L517.

BARKER, P. M., NGUYEN, M. S., GATZY, J. T., GRUBB, B., NORMAN, H., HUMMLER, E., ROSSIER, B., BOUCHER, R. C. & KOLLER, B. (1998). Role of gammaENaC subunit in lung liquid clearance and electrolyte balance in newborn mice. Insights into perinatal adaptation and pseudohypoaldosteronism. *J Clin Invest.,* **102:** 1634-40.

BARKER, P., M., STRANG, L., B., WALTERS, D., V. (1990). The role of thyroid hormones in maturation of the adrenaline-sensitive lung liquid reabsorptive mechanism in fetal sheep. *J Physiol.*, **424:** 473-85.

BARKER, P., M., WALTERS, D., V., MARKIEWICZ, M. & STRANG, L. B. (1991). Development of the lung liquid reabsorptive mechanism in fetal sheep: synergism of triidothyronine and hydrocortisone. *J Physiol.*, **433:** 435-49.

BARNES P.J. (2011). Glucocorticosteroids: current and future directions. *Br. J. Pharmacol.* 163, 29-43.

BARQUIN N, CICCOLELLA DE, RIDGE KM & SZNAJDER JI. (1997). Dexamethasone upregulates the Na-K-ATPase in rat alveolar epithelial cells, pp. L825. *Am Physiol Soc.*

BAYASCAS, J. R. & ALESSI, D. R. (2005). Regulation of Akt/PKB Ser4^{73} phosphorylation. *Mol Cell,* **18:** 143-5.

BEARD, L. L., LI, T., HU, Y. & FOLKESSON, H. G. (2011). Fetal Lung Epithelial Ion Channels Relocate in the Cell Membrane During Late Gestation. *Anat Rec (Hoboken).*, Aug 1 *Epub ahead of print*

BENOS D. J. (1982). Amiloride: A molecular probe of sodium transport in tissues and cells. *Am J of Physiol.*,**242:** 131–45.

BENOS, D. J. & STANTON, B. A. (1999). Functional domains within the degenerin/epithelial sodium channel (Deg/ENaC) superfamily of ion channels. *J of Physiol (Lond),* **520:** 631-644.

BERDIEV, B. K., KARLSON, K. H., JOVOV, B., RIPOLL, P. J., MORRIS, R., LOFFING-CUENI, D., HALPIN, P., STANTON, B. A., KLEYMAN, T. R. & ISMAILOV, I. I. (1998). Subunit stoichiometry of a core conduction element in a cloned epithelial amiloride-sensitive Na^+ channel. *Biophys J,* **75:** 2292-301.

BERGER PJ, SMOLICH JJ, RAMSDEN CA & WALKER AM. (1996). Effect of lung liquid volume on respiratory performance after caesarean delivery in the lamb, pp. 905. *Physiol Soc.*

BERGER, P.J., KYRIAKIDES, M.A., SMOLICH, J.J., RAMSDEN, C.A., WALKER, A.M. (1998). Massive decline in lung liquid before vaginal delivery at term in the fetal sheep. *Am. J. Obstet. Gynecol.* 178, 223–227.

BHALLA, V., DAIDIÉ, D., LI, H., PAO, A. C., LAGRANGE, L. P., WANG, J., VANDEWALLE, A., STOCKAND, J. D., STAUB, O. & PEARCE, D. (2005). Serum- and glucocorticoid-regulated kinase 1 regulates ubiquitin ligase neural precursor cell-expressed, developmentally down-regulated protein 4-2 by inducing interaction with 14-3-3. *Mol Endocrinol,* **19:** 3073-84.

BHALLA, V., OYSTER, N. M., FITCH, A. C., WIJNGAARDEN, M. A., NEUMANN, D., SCHLATTNER, U., PEARCE, D. & HALLOWS, K. R. (2006). AMP-activated kinase inhibits the epithelial Na^+ channel through functional regulation of the ubiquitin ligase Nedd4-2. *J Biol Chem,* **281:** 26159-69.

BIONDI, R. M., KIELOCH, A., CURRIE, R. A., DEAK, M. & ALESSI, D. R. (2001). The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J,* **20:** 4380-90.

BLAND R.D., BRESSACK M.A., MCMILLAN D. D. (1979). Labor decreases the lung water content of newborn rabbits. *Am J Obstet Gynecol.* **135(3):**364-7.

BLAND RD, MCMILLAN DD, BRESSACK MA & DONG L. (1980b). Clearance of liquid from lungs of newborn rabbits, pp. 171. *Am Physiol Soc.*

BLAZER-YOST, B. L., ESTERMAN, M. A. & VLAHOS, C. J. (2003). Insulin-stimulated trafficking of ENaC in renal cells requires PI 3-kinase activity. *Am J Physiol Cell Physiol,* **284:** 1645-53.

BLAZER-YOST, B. L., LIU, X. & HELMAN, S. I. (1998). Hormonal regulation of ENaCs: insulin and aldosterone. *Am J Physiol,* **274:** 1373-9.

BLAZER-YOST, B. L. & NOFZIGER, C. (2004). The role of the phosphoinositide pathway in hormonal regulation of the epithelial sodium channel. *Adv Exp Med Biol,* **559:** 359-68.

BLAZER-YOST, B. L. & NOFZIGER, C. (2005). Phosphoinositide lipid second messengers: new paradigms for transepithelial signal transduction. *Pflugers Arch,* **450:** 75-82.

BOASE, N. A., RYCHKOV, G. Y., TOWNLEY, S. L., DINUDOM, A., CANDI, E., VOSS, A. K., TSOUTSMAN, T., SEMSARIAN, C., MELINO, G., KOENTGEN, F., COOK, D. I. & KUMAR, S. (2011). Respiratory distress and perinatal lethality in Nedd4-2-deficient mice. *Nat Commun,* **2:** 287.

BOOTH, R. E. & STOCKAND, J. D. (2003). Targeted degradation of ENaC in response to PKC activation of the ERK1/2 cascade. *Am J Physiol Renal Physiol.,* **284:** 938-47.

BOROK, Z., LIEBLER, J. M., LUBMAN, R. L., FOSTER, M. J., ZHOU, B., LI, X., ZABSKI, S. M., KIM, K. J. & CRANDALL, E. D. (2002). Na^+ transport proteins are expressed by rat alveolar epithelial type I cells. *Am J Physiol Lung Cell Mol Physiol,* **282:** 599-608.

BOUCHER, R.C., (1999) Molecular insights into the physiology of the 'thin film' of airway surface liquid. *J Physiol (Lond),* **516:** 631-8.

BOURKE S, MASON HS, BOROK Z, KIM KJ, CRANDALL ED & KEMP PJ. (2005). Development of a lung slice preparation for recording ion channel activity in alveolar epithelial type I cells, pp. 40.

BOYD, C. & NÁRAY-FEJES-TÓTH, A. (2005). Gene regulation of ENaC subunits by serum- and glucocorticoid-inducible kinase-1. *Am J Physiol Renal Physiol,* **288:** 505-12.

BOYD, C. & TOTH, A. N. F. (2005). Gene regulation of ENaC subunits by serum- and glucocorticoid-inducible kinase-1. *Am J Physiol- Renal Physiol.,* **288:** 505-12.

BOYDEN EA, Ed: Development of the Lung: Lung Biology in Health and Disease. Dekker, NY; 1977.

BRIDGES, R. J., NEWTON, B. B., PILEWSKI, J. M., DEVOR, D. C., POLL, C. T. & HALL, R. L. (2001). Na^+ transport in normal and CF human bronchial epithelial cells is inhibited by BAY 39-9437. *Am J Physiol Lung Cell Mol Physiol,* **281:** 16-23.

BROWN, M. J., OLVER, R. E., RAMSDEN, C. A., STRANG, L. B. & WALTERS, D. V. (1983). Effects of Adrenaline and of spontaneous labor on the secretion and absorption of lung liquid in the fetal lamb. *J Physiol (Lond).,* **344:** 137-52.

BROWN, S. G., GALLACHER, M., OLVER, R. E. & WILSON, S. M. (2008). The regulation of selective and nonselective Na^+ conductances in H441 human airway epithelial cells. *Am J of Physiol - Lung Cell Mol Physiol.,* **294:** 942-54.

BRUCE, M. C., KANELIS, V., FOULADKOU, F., DEBONNEVILLE, A., STAUB, O. & ROTIN, D. (2008). Regulation of Nedd4-2 self-ubiquitination and stability by a PY motif located within its HECT-domain. *Biochem J.,* **415:** 155-63.

BRUNS, J. B., CARATTINO, M. D., SHENG, S. H., MAAROUF, A. B., WEISZ, O. A., PILEWSKI, J. M., HUGHEY, R. P. & KLEYMAN, T. R. (2007). Epithelial Na^+ channels are fully activated by furin- and prostasin-dependent release of an inhibitory peptide from the gamma-subunit. *J Biol Chem.,* **282:** 6153-60.

BRUNS, J. B., HU, B. F., AHN, Y. J., SHENG, S. H., HUGHEY, R. P. & KLEYMAN, T. R. (2003). Multiple epithelial Na^+ channel domains participate in subunit assembly. *Am J Physiol - Renal Physiol.,* **285:** 600-09.

BUCHER U. & REID L. (1961). Development of the intrasegmental bronchial tree: The pattern of branching and development of cartilage at various stages of intra-uterine life. *Thorax* **16:** 207-218.

BUREN J, LAI YC, LUNDGREN M, ERIKSSON JW & JENSEN J. (2008). Insulin action and signalling in fat and muscle from dexamethasone-treated rats, pp. 91-101. Elsevier.

BURRI, P.H. (1984) Fetal and postnatal development of the lung. *Ann Rev Physiol* 46: 617-28

BURRI, P.H. (1985) Chapter 1: Development and growth of the human lung. Handbook of Physiology – Respiratory System. Pg 1-46. Bethesda: *Am Physiol Soc*, USA

BURRI PH (1988) Development and regeneration of the lung. In: Fishman AP (ed): Pulmonary Diseases and Disorders. Vol. 1. New York: McGraw-Hill, pp. 61 -78.

BUTTERWORTH, M. B. (2010). Regulation of the epithelial sodium channel (ENaC) by membrane trafficking. *Biochim Biophys Acta,* **1802:** 1166-77.

BUTTERWORTH, M. B., EDINGER, R. S., FRIZZELL, R. A. & JOHNSON, J. P. (2009). Regulation of the epithelial sodium channel by membrane trafficking. *Am J Physiol - Renal Physiol.,* **296:** 10-24.

BUTTERWORTH, M. B., EDINGER, R. S., JOHNSON, J. P. & FRIZZELL, R. A. (2005). Acute ENaC stimulation by cAMP in a kidney cell line is mediated by exocytic insertion from a recycling channel pool. *J Gen Physiol,* **125:** 81-101.

CALDWELL R.A, BOUCHER R.C, STUTTS M.J. (2004). Serine protease activation of near-silent epithelial Na^+ channels. *Am. J. Physiol.-Cell Physiol.* 286, C190-C194.

CANESSA, C. M. (1996). What is new about the structure of the epithelial Na^+ channel? *News Physiol Sci* **11:** 195—201.

CANESSA, C. M., HORISBERGER, J. D. & ROSSIER, B. C. (1993). Epithelial sodium channel related to proteins involved in neurodegeneration. *Nature,* **361:** 467-70.

CANESSA, C. M., HORISBERGER, J. D., SCHILD, L. & ROSSIER, B. C. (1995). Expression cloning of the epithelial sodium channel. *Kidney Int,* **48:** 950-5.

CANESSA, C. M., SCHILD, L., BUELL, G., THORENS, B., GAUTSCHI, I., HORISBERGER, J. D. & ROSSIER, B. C. (1994). Amiloride-sensitive epithelial Na^+ channel is made of 3 homologous subunits. *Nature,* **367:** 463-467.

CARATTINO, M. D., SHENG, S. H., BRUNS, J. B., PILEWSKI, J. M., HUGHEY, R. P. & KLEYMAN, T. R. (2006). The epithelial Na^+ channel is inhibited by a peptide derived from proteolytic processing of its alpha subunit. *J Biol Chem.,* **281:** 18901-07.

CARLTON, D.P., CUMMINGS, J.J., CHAPMAN, D.L., POULAIN, F.R., BLAND, R.D. (1992a). Ion transport regulation of lung liquid secretion in foetal lambs. *J. Dev. Physiol.* 17, 99–107.

CARLTON, D.P., CUMMINGS, J.J., POULAIN, F.R., BLAND, R.D. (1992b). Increased pulmonary vascular filtration pressure does not alter lung liquid secretion in fetal sheep. *J. Appl. Physiol.* 72 (2), 650–655.

CASSIN, S., GAUSE, G. & PERKS, A. (1986). The effects of bumetanide and furosemide on lung liquid secretion in fetal sheep. *Proc. Soc Exp Biol Med*, **181:** 427-431.

CASTRANOVA V, RABOVSKY J, TUCKER JH, MILES PR (1988). The alveolar type II epithelial cells : a multifunctional pneumocyte. *Toxicol Appl Pharmacol.* **93**:472-83.

CHALFANT M.L., DENTON J.S., LANGLOH A.L., KARLSON K.H., LOFFING J., BENOS D.J., STANTON B.A. (1999). The NH_2 terminus of the epithelial sodium channel contains an endocytic motif. *J Biol Chem*. **274:** 32889-96.

CHAN, T. O., RITTENHOUSE, S. E. & TSICHLIS, P. N. (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem,* **68:** 965-1014.

CHANDRAN, S., LI, H., DONG, W., KRASINSKA, K., ADAMS, C., ALEXANDROVA, L., CHIEN, A., HALLOWS, K. R. AND BHALLA, V. (2011) Neural Precursor Cell-expressed Developmentally Down-regulated Protein 4-2 (Nedd4-2) Regulation by 14-3-3 Protein Binding at Canonical Serum and Glucocorticoid Kinase 1 (SGK1) Phosphorylation Sites. *J. Biol. Chem.* 286, 37830-37840

CHEN, H. I. & SUDOL, M. (1995). The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. *Proc Natl Acad Sci U S A,* **92:** 7819-23.

CHEN, S. Y., BHARGAVA, A., MASTROBERARDINO, L., MEIJER, O. C., WANG, J., BUSE, P., FIRESTONE, G. L., VERREY, F. & PEARCE, D. (1999). Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc Natl Acad Sci U S A*, **96:** 2514-9.

CHENG, C., PRINCE, L. S., SNYDER, P. M. & WELSH, M. J. (1998). Assembly of the epithelial Na^+ channel evaluated using sucrose gradient sedimentation analysis. *J Biol Chem*, **273:** 22693-700.

CHRIST-CRAIN,M. KOLA, B., LOLLI, F. KORBONITS, M. (2008). AMP-activated protein kinase mediates glucocorticoidinduced metabolic changes: a novel mechanism in Cushing's syndrome. *The FASEB J* doi: 10.1096/fj.07-094144.

CLUNES, M. T., BUTT, A. G. & WILSON, S. M. (2004). A glucocorticoid-induced Na^+ conductance in human airway epithelial cells identified by perforated patch recording. *J Physiol (Lond)*, **557:** 809-19.

COLLETT A, RAMMINGER SJ, OLVER RE & WILSON SM. (2002). -Adrenoceptor-mediated control of apical membrane conductive properties in fetal distal lung epithelia, pp.L621. *Am Physiol Soc*.

CUTOLO M, VILLAGGIO B, BRIZZOLARA R, MONTAGNA P, GALLO F, MORETTI S, BONASSI S, SULLI A & SOLDANO S. (2011). Identification and quantification of selected inflammatory genes modulated by leflunomide and prednisone treatment in early rheumatoid arthritis patients, pp. 72-79. *Clin Exp Rheumatol.*

DAGENAIS, A., FRECHETTE, R., CLERMONT, M. E., MASSE, C., PRIVE, A., BROCHIERO, E. & BERTHIAUME, Y. (2006). Dexamethasone inhibits the action of TNF on ENaC expression and activity. *Am J Physiol - Lung Cell Mol Physiol.,* **291:** 1220-31.

DEBONNEVILLE, C., FLORES, S. Y., KAMYNINA, E., PLANT, P. J., TAUXE, C., THOMAS, M. A., MUNSTER, C., CHRAIBI, A., PRATT, J. H., HORISBERGER, J. D., PEARCE, D., LOFFING, J. & STAUB, O. (2001). Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na^+ channel cell surface expression. *Embo Journal,* **20:** 7052-59.

DENG, W., WANG, D. X., ZHANG, W. & LI, C. Y. (2011). Regulation of epithelial sodium channel α-subunit expression. *Chin Med J (Engl),* **124:** 1551-5.

DIAKOV, A., BERA, K., MOKRUSHINA, M., KRUEGER, B. & KORBMACHER, C. (2008). Cleavage in the gamma-subunit of the epithelial sodium channel (ENaC) plays an important role in the proteolytic activation of near-silent channels. *J Physiol (Lond)*, **586:** 4587-4608.

DIAKOV, A. & KORBMACHER, C. (2004). A novel pathway of epithelial sodium channel activation involves a serum- and glucocorticoid-inducible kinase consensus motif in the C terminus of the channel's alpha-subunit. *J Biol Chem.,* **279:** 38134-142.

DIAKOV, A., NESTEROV, V., MOKRUSHINA, M., RAUH, R. & KORBMACHER, C. (2010). Protein kinase B alpha (PKBα) stimulates the epithelial sodium channel (ENaC) heterologously expressed in Xenopus laevis oocytes by two distinct mechanisms. *Cell Physiol Biochem,* **26:** 913-24.

DINUDOM, A., HARVEY, K. F., KOMWATANA, P., JOLLIFFE, C. N., YOUNG, J. A., KUMAR, S. & COOK, D. I. (2001). Roles of the C termini of alpha-, beta-, and gamma-subunits of epithelial Na^+ channels (ENaC) in regulating ENaC and mediating its inhibition by cytosolic Na^+. *J Biol Chem.*, **276:** 13744-49.

DOBBS, L. G., R. GONZALEZ, M. A. MATTHAY, E. P. CARTER, L. ALLEN & A. S. VERKMAN (1998). Highly water-permeable type I alveolar epithelial cells confer high water permeability between the airspace and vasculature in rat lung. *Proc Natl Acad Sci USA,* **95**: 2991-6.

EATON DC, HELMS MN, KOVAL M, BAO HF & JAIN L. (2006). The contribution of epithelial sodium channels to alveolar function in health and disease, pp. 403-423.Annual Reviews.

EATON, D.C., MALIK, B., BAO, H. F., YU, L. & JAIN, L. (2010). Regulation of epithelial sodium channel trafficking by ubiquitination. *Proc Am Thorac Soc*, **7:** 54-64.

EBRAHIMI, S., SARKARI, B. (2007). Comparative efficacy of dexamethasone versus hydrocortisone in severe acute pediatric asthma. *Iran J Allergy Asthma Immunol.* **6(3)**: 159-160.

EGLI, M., DUPLAIN, F., LEPORI, M., COOK, S., NICOD, P., HUMMLER, E., SARTORI, C. & SCHERRER, U. (2004). Defective respiratory amiloride-sensitive sodium transport predisposes to pulmonary oedema and delays its resolution in mice. *J Physiol (Lond),* **560:** 857-865.

EMBARK HM, BÖHMER C, PALMADA M, RAJAMANICKAM J, WYATT AW, WALLISCH S, CAPASSO G, WALDEGGER P, SEYBERTH HW, WALDEGGER S, LANG F. (2004). Regulation of CLC-Ka/barttin by the ubuquitin ligase and the serum and glucocorticoid dependent kinases. *Kidney Int* **66(5):**1918-25.

ERGONUL, Z., FRINDT, G. & PALMER, L. G. (2006). Regulation of maturation and processing of ENaC subunits in the rat kidney. *Am J Physiol - Renal Physiol,* **291:** 683-93.

ESKANDARI, S., SNYDER, P. M., KREMAN, M., ZAMPIGHI, G. A., WELSH, M. J. & WRIGHT, E. M. (1999). Number of subunits comprising the epithelial sodium channel. *J Biol Chem*, **274:** 27281-6.

FALETTI, C. J., PERROTTI, N., TAYLOR, S. I. & BLAZER-YOST, B. L. (2002). sgk: an essential convergence point for peptide and steroid hormone regulation of ENaC-mediated Na^+ transport. *Am J Physiol Cell Physiol,* **282:** 494-500.

FARMAN, N., TALBOT, C., BOUCHER, R., FAY, M., CANESSA, C., ROSSIER, B. AND BONVALET, J.P. (1997). Noncoordinated expression of alpha-, beta-, and gamma-subunit mRNAs of epithelial Na^+ channel along rat respiratory tract. *Am. J. Physiol.* **272**, C131-141.

FARR, T. J., CODDINGTON-LAWSON, S. J., SNYDER, P. M. & MCDONALD, F. J. (2000). Human Nedd4 interacts with the human epithelial Na^+ channel: WW3 but not WW1 binds to Na^+-channel subunits. *Biochem J*, **345:** 503-9.

FAURE-FREMIET & DRAGOIU. (1923). Le developpement du poumon foetal chez le mouton. *Arch Anat Micr,* **19:** 411-474.

FEHRENBACH H: Alveolar epithelia type II cell: Defender of the alveolus revisited. *Respir Res* 2001, 2:33-46

FELDMAN ME, APSEL B, UOTILA A, LOEWITH R, KNIGHT ZA, RUGGERO D & SHOKAT KM. (2009). Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2, pp. e1000038.

FEJES-TOTH, G., FRINDT, G., NARAY-FEJES-TOTH, A. & PALMER, L. G. (2008). Epithelial Na^+ channel activation and processing in mice lacking SGK1. *Am J Physiol-Renal Physiol.,* **294:** 1298-1305.

FINGAR DC, RICHARDSON CJ, TEE AR, CHEATHAM L, TSOU C & BLENIS J. (2004). mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4EBP1/ eukaryotic translation initiation factor 4E, pp. 200. *Am Soc Microbiol.*

FIRSOV, D., GAUTSCHI, I., MERILLAT, A., ROSSIER, B. & SCHILD, L. (1998). The heterotetrameric architecture of the epithelial sodium channel (ENaC). *Embo J***, 17:** 344-52.

FIRSOV D, SCHILD L, GAUTSCHI I, MÉRILLAT AM, SCHNEEBERGER E, ROSSIER BC. (1996) Cell surface expression of the epithelial Na channel and a mutant causing Liddle syndrome: a quantitative approach. *Proc Natl Acad Sci USA*. **93(26):**15370–5.

FLORES, S. Y., LOFFING-CUENI, D., KAMYNINA, E., DAIDIÉ, D., GERBEX, C., CHABANEL, S., DUDLER, J., LOFFING, J. & STAUB, O. (2005). Aldosterone-induced serum and glucocorticoid-induced kinase 1 expression is accompanied by Nedd4-2 phosphorylation and increased Na^+ transport in cortical collecting duct cells. *J Am Soc Nephrol,* **16:** 2279-87.

FÖDINGER M., HIRSCHL M.M., SCHEDLER D., HERKNER H., BUR A., LAGGNER A.N., HÖRL W.H., SUNDER-PLASSMANN G. (1998). Mutations in the carboxy terminus of the beta and gamma subunits of the epithelial sodium channel are not present in patients with hypertensive crisis. *Eur J Clin Invest.* **28(9):** 707-11.

FOLKESSON, H. G., NORLIN, A., WANG, Y. B., ABEDINPOUR, P. & MATTHAY, M. A. (2000). Dexamethasone and thyroid hormone pretreatment upregulate alveolar epithelial fluid clearance in adult rats. *J Appl Physiol.*, **88:** 416-24.

FONSECA, B. D., SMITH, E. M., LEE, V. H. Y., MACKINTOSH, C. & PROUD, C. G. (2007). PRAS40 is a target for mammalian target of rapamycin complex 1 and is required for signaling downstream of this complex. *J Biol Chem.,* **282:** 24514-24.

FOTIA, A. B., DINUDOM, A., SHEARWIN, K. E., KOCH, J. P., KORBMACHER, C., COOK, D. I. & KUMAR, S. (2003). The role of individual Nedd4-2 (KIAA0439) WW domains in binding and regulating epithelial sodium channels. *FASEB J,* **17:** 70-2.

FRIEDRICH, B., FENG, Y., COHEN, P., RISLER, T., VANDEWALLE, A., BROER, S., WANG, J., PEARCE, D. & LANG, F. (2003). The serine/threonine kinases SGK2 and SGK3 are potent stimulators of the epithelial Na^+ channel alpha,beta,gamma-ENaC. *Pflugers Arch-Eur J Physiol.,* **445:** 693-696.

FRINDT, G., MASILAMANI, S., KNEPPER, M. A. & PALMER, L. G. (2001). Activation of epithelial Na channels during short-term Na deprivation. *Am J Physiol Renal Physiol,* **280:** 112-8.

FYFE, G. K. & CANESSA, C. M. (1998). Subunit composition determines the single channel kinetics of the epithelial sodium channel. *J Gen Physiol,* **112:** 423-32.

GAEGGELER, H. P., GUILLOD, Y., LOFFING-CUENI, D., LOFFING, J. & ROSSIER, B. C. (2011). Vasopressin-dependent coupling between sodium transport and water flow in a mouse cortical collecting duct cell line. *Kidney Int,* **79:** 843-52.

GALLACHER, M. (2009). *The control of membrane Na^+ conductance in normal and influenza A virus infected epithelial cells.* PhD, University of Dundee.

GALLACHER M, BROWN SG, HALE BG, FEARNS R, OLVER RE, RANDALL RE & WILSON SM. (2009). Cation currents in human airway epithelial cells induced by infection with influenza A virus, pp. 3159-3173. Wiley Online Library.

GAO, P. J., ZHANG, K. X., ZHU, D. L., HE, X., HAN, Z. Y., ZHAN, Y. M. & YANG, L. W. (2001). Diagnosis of Liddle syndrome by genetic analysis of beta and gamma subunits of epithelial sodium channel - a report of five affected family members. *J Hypertens,* **19:** 885-9.

GARCIA-MARTINEZ, J. M. & ALESSI, D. R. (2008). mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochem J,* **416:** 375-85.

GARTY, H. & PALMER, L. (1997). Epithelial sodium channels: Function, structure, and regulation. *Physiol Rev***, 77:** 359-96.

GAZDAR, A.F., LINNOILA R.I., KURITA Y. (1990) Peripheral airway cell differentiation in human lung cancer cell lines. *Cancer Res*, **50:** 5481-87.

GONZALEZ-ROBAYNA, I. J., FALENDER, A. E., OCHSNER, S., FIRESTONE, G. L. AND RICHARDS, J. S. (2000) Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): Evidence for A kinase-independent signaling by FSH in granulosa cells. *Mol. Endocrinol.* 14, 1283-1300

GONZALEZ-RODRIGUEZ E, GAEGGELER HP & ROSSIER BC. (2007). IGF-1 vs insulin: respective roles in modulating sodium transport via the PI-3 kinase/Sgk1 pathway in acortical collecting duct cell line, pp. 116-125. *Nature Publishing Group.*

GOULET, C. C., VOLK, K. A., ADAMS, C. M., PRINCE, L. S., STOKES, J. B. & SNYDER, P. M. (1998). Inhibition of the epithelial Na^+ channel by interaction of Nedd4 with a PY motif deleted in Liddle's syndrome. *Journal of Biological Chemistry,* **273:** 30012-17.

GREENWOOD, I. A., YEUNG, S. Y., HETTIARACHI, S., ANDERSSON, M. & BAINES, D. L. (2009). KCNQ-encoded channels regulate Na^+ transport across H441 lung epithelial cells. *Pflugers Arch,* **457:** 785-94.

GRÜNDER, S., FIRSOV, D., CHANG, S. S., JAEGER, N. F., GAUTSCHI, I., SCHILD, L., LIFTON, R. P. & ROSSIER, B. C. (1997). A mutation causing pseudohypoaldosteronism type 1 identifies a conserved glycine that is involved in the gating of the epithelial sodium channel. *EMBO J,* **16:** 899-907.

GUAZZI, M., BRAMBILLA, R., DE VITA, & GUAZZI, M. D. (2002a). Diabetes Worsens Pulmonary Diffusion in Heart Failure, and Insulin Counteracts This Effect. *Am J Respir Crit Care Med*, **166:** 978-82.

GUAZZI, M., OREGLIA, I. & GUAZZI, M. D. (2002b) Insulin Improves Alveolar Capillary Membrane Gas Conductance in Type 2 Diabetes. *Diabetes Care*, **25:** 1802-06.

GUERTIN, D. A. & SABATINI, D. M. (2007). Defining the role of mTOR in cancer. *Cancer Cell,* **12:** 9-22.

GUNEY, S., SCHULER, A., OTT, A., HOSCHELE, S., ZUGEL, S., BALOGLU, E., BARTSCH, P. & MAIRBAURL, H. (2007). Dexamethasone prevents transport inhibition by hypoxia in rat lung and alveolar epithelial cells by stimulating activity and expression of Na^+-K^+-ATPase and epithelial Na^+ channels. *Am J Physiol - Lung Cell Mol Physiol.*, **293:** 1332-38.

HADDARD J. J. (2002) Oxygen-sensing mechanisms and the regulation of redox-responsive transcription factors in development and pathophysiology. *Resp Res* **3**:26

HAEFELI-BLEUER B, WEIBEL ER: Morphometry of the human pulmonary acinus. *Anat Rec* 220 :401, 1988.

HAGIWARA, N., TOHDA, H., DOI, Y., O'BRODOVICH, H. & MARUNAKA, Y. (1992). Effects of insulin and tyrosine kinase inhibitor on ion transport in the alveolar cell of the fetal lung. *Biochem Biophys Res Commun,* **187:** 802-8.

HALLOWS, K. R., BHALLA, V., OYSTER, N. M., WIJNGAARDEN, M. A., LEE, J. K., LI, H., CHANDRAN, S., XIA, X., HUANG, Z., CHALKLEY, R. J., BURLINGAME, A. L. & PEARCE, D. (2010). Phosphopeptide screen uncovers novel phosphorylation sites of Nedd4-2 that potentiate its inhibition of the epithelial Na^+ channel. *J Biol Chem,* **285:** 21671-8.

HANSSON, J. H., NELSON-WILLIAMS, C., SUZUKI, H., SCHILD, L., SHIMKETS, R., LU, Y., CANESSA, C., IWASAKI, T., ROSSIER, B. & LIFTON, R. P. (1995a). Hypertension caused by a truncated epithelial sodium channel gamma subunit: genetic heterogeneity of Liddle syndrome. *Nat Genet,* **11:** 76-82.

HANSSON, J. H., SCHILD, L., LU, Y., WILSON, T. A., GAUTSCHI, I., SHIMKETS, R., NELSON-WILLIAMS, C., ROSSIER, B. C. & LIFTON, R. P. (1995b). A de novo missense mutation of the beta subunit of the epithelial sodium channel causes hypertension and Liddle syndrome, identifying a proline-rich segment critical for regulation of channel activity. *Proc Natl Acad Sci USA,***92:**11495-9.

HANWELL, D., ISHIKAWA, T., SALEKI, R. & ROTIN, D. (2002). Trafficking and cell surface stability of the epithelial Na^+ channel expressed in epithelial Madin-Darby canine kidney cells. *J Biol Chem,* **277:** 9772-9.

HARA K, YONEZAWA K, KOZLOWSKI MT, SUGIMOTO T, ANDRABI K, WENG QP, KASUGA M, NISHIMOTO I & AVRUCH J. (1997). Regulation of eIF-4E BP1 phosphorylation by mTOR, pp. 26457. ASBMB.

HARDING R & HOOPER S.B. (1996) Regulation of lung expansion and lung growth before birth. *J Appl Physiol.* **81 (1):** 209-224

HARLOW, E. & LANE, D. 1988. *Antibodies: A Laboratory Manual,* New York, USA, Cold Spring Harbor Laboratory.

HARRIS, M., FIRSOV, D., VUAGNIAUX, G., STUTTS, M. J. & ROSSIER, B. C. (2007). A novel neutrophil elastase inhibitor prevents elastase activation and surface cleavage of the epithelial sodium channel expressed in Xenopus laevis oocytes. *J Biol Chem,* **282:** 58-64.

HARVEY, K. F., DINUDOM, A., COOK, D. I. & KUMAR, S. (2001). The Nedd4-like protein KIAA0439 is a potential regulator of the epithelial sodium channel. *J Biol Chem,* **276:** 8597-601.

HEISE, C. J., XU, B. E., DEATON, S. L., CHA, S. K., CHENG, C. J., EARNEST, S., SENGUPTA, S., JUANG, Y. C., STIPPEC, S., XU, Y., ZHAO, Y., HUANG, C. L. & COBB, M. H. (2010). Serum and glucocorticoid-induced kinase (SGK) 1 and the epithelial sodium channel are regulated by multiple with no lysine (WNK) family members. *J Biol Chem,* **285:** 25161-7.

HELMS, M. N., FEJES-TOTH, G. & NARAY-FEJES-TOTH, A. (2003). Hormone-regulated transepithelial Na^+ transport in mammalian CCD cells requires SGK1 expression. *Am J Physiol Renal Physiol,* **284:** 480-7.

HELMS MN, SELF J, BAO HF, JOB LC, JAIN L & EATON DC. (2006). Dopamine activates amiloride-sensitive sodium channels in alveolar type I cells in lung slice preparations, pp. L610. Am Physiological Soc.

HELVE, O., PITKÄNEN, O. M., ANDERSSON, S., O'BRODOVICH, H., KIRJAVAINEN, T. & OTULAKOWSKI, G. (2004). Low expression of human epithelial sodium channel in airway epithelium of preterm infants with respiratory distress. *Pediatrics,* **113:** 1267-72.

HENRY, P. C., KANELIS, V., O'BRIEN, M. C., KIM, B., GAUTSCHI, I., FORMAN-KAY, J., SCHILD, L. & ROTIN, D. (2003). Affinity and specificity of interactions between Nedd4 isoforms and the epithelial Na^+ channel. *J Biol Chem,* **278:** 20019-28.

HONG F, LARREA MD, DOUGHTY C, KWIATKOWSKI DJ, SQUILLACE R & SLINGERLAND JM. (2008). mTOR-raptor binds and activates SGK1 to regulate p27 phosphorylation, pp.701-711. Elsevier.

HOOPER, J. D., CLEMENTS, J. A., QUIGLEY, J. P. & ANTALIS, T. M. (2001). Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. *J Biol Chem,* **276:** 857-60.

HORISBERGER, J. D. (1998). Amiloride-sensitive Na^+ channels. *Curr Opin Cell Biol,* **10:** 443-9.

HU J.C., BENGRINE A., LIS A., AWAYDA M.S. (2009). Alternative mechanism of activation of the epithelial Na^+ channel by cleavage., **284:** 36334-45.

HUGHEY, R. P., BRUNS, J. B., KINLOUGH, C. L., HARKLEROAD, K. L., TONG, Q. S., CARATTINO, M. D., JOHNSON, J. P., STOCKAND, J. C. & KLEYMAN, T. R. (2004a). Epithelial sodium channels are activated by furin-dependent proteolysis. *J Biol Chem,* **279:** 18111-14.

HUGHEY, R. P., BRUNS, J. B., KINLOUGH, C. L. & KLEYMAN, T. R. (2004b). Distinct pools of epithelial sodium channels are expressed at the plasma membrane. *J Biol Chem,* **279:** 48491-4.

HUGHEY, R. P. & KLEYMAN, T. R. (2007). Functional cross talk between ENaC and pendrin. *Am J Physiol Renal Physiol,* **293:** 1439-40.

HUGHEY, R. P., MUELLER, G. M., BRUNS, J. B., KINLOUGH, C. L., POLAND, P. A., HARKLEROAD, K. L., CARATTINO, M. D. & KLEYMAN, T. R. (2003). Maturation of the epithelial Na^+ channel involves proteolytic processing of the alpha- and gamma-subunits. *J Biol Chem,* **278:** 37073-82.

HUIBREGTSE, J. M., SCHEFFNER, M., BEAUDENON, S. & HOWLEY, P. M. (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci U S A,* **92:** 5249.

HUMMLER, E., BARKER, P., GATZY, J., BEERMANN, F., VERDUMO, C., SCHMIDT, A., BOUCHER, R. & ROSSIER, B. C. (1996). Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. *Nat Genet,* **12:** 325-8.

HUSBAND, E. M. (2006). *Regulation of Na^+ transport in H441 human bronchiolar epithelial cells.* PhD. University of Dundee

ICHIMURA, T., YAMAMURA, H., SASAMOTO, K., TOMINAGA, Y., TAOKA, M., KAKIUCHI, K., SHINKAWA, T., TAKAHASHI, N., SHIMADA, S. & ISOBE, T. (2005). 14-3-3 proteins modulate the expression of epithelial Na^+ channels by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase. *J Biol Chem,* **280:** 13187-94.

ILSLEY, J. L., SUDOL, M. & WINDER, S. J. (2002). The WW domain: linking cell signalling to the membrane cytoskeleton. *Cell Signal,* **14:** 183-9.

INGLIS, S. K., GALLACHER, M., BROWN, S. G., MCTAVISH, N., GETTY, J., HUSBAND, E. M., MURRAY, J. T. & WILSON, S. M. (2009). SGK1 activity in Na^+ absorbing airway epithelial cells monitored by assaying NDRG1-Thr($^{346/356/366}$) phosphorylation. *Pflugers Arch - Eur J Physiol,* **457:** 1287-301.

ISMAILOV, I. I., KIEBER-EMMONS, T., LIN, C., BERDIEV, B. K., SHLYONSKY, V. G., PATTON, H. K., FULLER, C. M., WORRELL, R., ZUCKERMAN, J. B., SUN, W., EATON, D. C., BENOS, D. J. & KLEYMAN, T. R. (1997). Identification of an amiloride binding domain within the alpha-subunit of the epithelial Na^+ channel. *J Biol Chem,* **272:** 21075-83.

ITANI, O. A., AUERBACH, S. D., HUSTED, R. F., VOLK, K. A., AGELOFF, S., KNEPPER, M. A., STOKES, J. B. & THOMAS, C. P. (2002a). Alveolar epithelial ion and fluid transport - Glucocorticoid-stimulated lung epithelial Na^+ transport is associated with regulated ENaC and sgk1 expression. *Am J Physiol - Lung Cell Mol Physiol,* **282:** 631-41.

ITANI, O. A., CAMPBELL, J. R., HERRERO, J., SNYDER, P. M. & THOMAS, C. P. (2003). Alternate promoters and variable splicing lead to hNedd4-2 isoforms with a C2 domain and varying number of WW domains. *Am J Physiol - Renal Physiol.,* **285:** 916-29.

ITANI, O. A., LIU, K. Z., CORNISH, K. L., CAMPBELL, J. R. & THOMAS, C. P. (2002b). Glucocorticoids stimulate human sgk1 gene expression by activation of a GRE in its 5 '-flanking region. *Am J Physiol-Endocrinol Met,* **283:** 971-79.

ITANI, O. A., STOKES, J. B. & THOMAS, C. P. (2005). Nedd4-2 isoforms differentially associate with ENaC and regulate its activity. *Am J Physiol-Renal Physiol.,* **289:** 334-46.

JACINTO E, FACCHINETTI V, LIU D, SOTO N, WEI S, JUNG SY, HUANG Q, QIN J & SU B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity, pp. 125-137. Elsevier.

JAIN, L., CHEN, X. J., MALIK, B., AL-KHALILI, O. & EATON, D. C. (1999). Antisense oligonucleotides against the alpha-subunit of ENaC decrease lung epithelial cation-channel activity. *Am J Physiol,* **276:** 1046-51.

JAIN, L., CHEN, X. J., RAMOSEVAC, S., BROWN, L. A. & EATON, D. C. (2001). Expression of highly selective sodium channels in alveolar type II cells is determined by culture conditions. *Am J Physiol Lung Cell Mol Physiol,* **280:** 646-58.

JANÉR, C., HELVE, O., PITKÄNEN, O. M., KARI, M. A., PELTONIEMI, O. M., HALLMAN, M., HOVI, P. & ANDERSSON, S. (2010). Expression of airway epithelial sodium channel in the preterm infant is related to respiratory distress syndrome but unaffected by repeat antenatal beta-methasone. *Neonatol,* **97:** 132-8.

JASTI, J., FURUKAWA, H., GONZALES, E. B. & GOUAUX, E. (2007). Structure of acid-sensing ion channel 1 at 1.9 A resolution and low pH. *Nature,* **449:** 316-23.

JOHNSON, M. D., BAO, H. F., HELMS, M. N., CHEN, X. J., TIGUE, Z., JAIN, L., DOBBS, L. G. & EATON, D. C. (2006). Functional ion channels in pulmonary alveolar type I cells support a role for type I cells in lung ion transport. *Proc Natl Acad Sci USA,* **103:** 4964-9.

JOHNSON, M. D., WIDDICOMBE, J. H., ALLEN, L., BARBRY, P. & DOBBS, L. G. (2002). Alveolar epithelial type I cells contain transport proteins and transport sodium, supporting an active role for type I cells in regulation of lung liquid homeostasis. *Proc Natl Acad Sci USA,* **99:** 1966-71.

JOST, A., POLICARD, A. (1948). Contribution exp´erimentale a l´´etude du d´eveloppement pr´enatal du poumon chez le lapin. *Arch. Anat. Microsc.* 37, 323–332.

JUNQUEIRA, L. C. U., J. CARNEIRO AND R. O. KELLEY (1998). *Basic Histology*. **pp 338**. Appleton & Lange, Norwalk, CT.

KABRA, R., KNIGHT, K. K., ZHOU, R. & SNYDER, P. M. (2008). Nedd4-2 induces endocytosis and degradation of proteolytically cleaved epithelial Na^+ channels. *J Biol Chem,* **283:** 6033-9.

KAMYNINA, E., DEBONNEVILLE, C., BENS, M., VANDEWALLE, A. & STAUB, O. (2001a). A novel mouse Nedd4 protein suppresses the activity of the epithelial Na^+ channel. *Faseb Journal,* **15:** 204-14.

KAMYNINA, E., DEBONNEVILLE, C., HIRT, R. P. & STAUB, O. (2001b). Liddle's syndrome: A novel mouse Nedd4 isoform regulates the activity of the epithelial Na^+ channel. *Kidney International,* **60:** 466-71.

KAMYNINA, E. & STAUB, O. (2002). Concerted action of ENaC, Nedd4-2, and Sgk1 in transepithelial $Na(^+)$ transport. *Am J Physiol Renal Physiol,* **283:** 377-87.

KAMYNINA, E., TAUXE, C. & STAUB, O. (2001c). Distinct characteristics of two human Nedd4 proteins with respect to epithelial Na^+ channel regulation. *Am J Physiol-Renal Physiol,* **281:** 469-77.

KASHLAN, O. B., SHENG, S. H. & KLEYMAN, T. R. (2005). On the interaction between amiloride and its putative alpha-subunit epithelial Na^+ channel binding site. *J Biol Chem,* **280:** 26206-15.

KASHLAN O.B., ADELMAN J.L., OKUMURA S., BLOBNER B.M., ZUZEK Z., HUGHEY R.P., KLEYMAN T.R., GRABE M. (2011) Constraint-based, homology model of the extracellular domain of the epithelial Na^+ channel α subunit reveals a mechanism of channel activation by proteases. *J Biol Chem* **286:** 649-60.

KASPER M, REIMANN T, HEMPEL U,WENZEL KW, BIERHAUS A, SCHUH D, DIMMER V, HAROSKE G, MULLER M (1998) Loss of caveolin expression in type I pneumocytes as an indicator of subcellular alterations during lung fibrogenesis. Histochem Cell Biol 109(1):41–48

KELLENBERGER, S., GAUTSCHI, I. & SCHILD, L. (1999a). A single point mutation in the pore region of the epithelial Na^+ channel changes ion selectivity by modifying molecular sieving. *Proc Natl Acad Sci USA,* **96:** 4170-5.

KELLENBERGER, S., HOFFMANN-POCHON, N., GAUTSCHI, I., SCHNEEBERGER, E. & SCHILD, L. (1999b). On the molecular basis of ion permeation in the epithelial Na^+ channel. *J Gen Physiol,* **114:** 13-30.

KELLENBERGER, S. & SCHILD, L. (2002). Epithelial sodium channel/degenerin family of ion channels: A variety of functions for a shared structure. *Physiol Rev,* **82:** 735-767.

KIEBER-EMMONS T., LIN C., FOSTER M.H., KLEYMAN T.R. (1999). Antiidiotypic antibody recognizes an amiloride binding domain within the alpha subunit of the epithelial Na^+ channel. *J Biol Chem* **274:** 9648-55.

KIKKAWA Y & YONEDA K. (1974). The type II epithelial cell of the lung. I. Method of isolation, pp. 76.

KIM D.H., SARBASSOV D.D, ALI S.M, KING J.E, LATEK R.R, ERDJUMENT-BROMAGE H, TEMPST P & SABATINI DM. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery, pp. 163-175. Elsevier.

KIM D.H, SARBASSOV D.D, ALI S.M, LATEK R.R, GUNTUR K.V.P, ERDJUMENT-BROMAGE H, TEMPST P & SABATINI DM. (2003). G [beta] L, a positive regulator of the rapamycinsensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR, pp. 895-904. Elsevier.

KIM, Y. H., PECH, V., SPENCER, K. B., BEIERWALTES, W. H., EVERETT, L. A., GREEN, E. D., SHIN, W., VERLANDER, J. W., SUTLIFF, R. L. & WALL, S. M. 2007. Reduced ENaC protein abundance contributes to the lower blood pressure observed in pendrin-null mice. *Am J Physiol Renal Physiol,* **293:** 1314-24.

KIMURA, T., KAWABE, H., JIANG, C., ZHANG, W., XIANG, Y. Y., LU, C., SALTER, M. W., BROSE, N., LU, W. Y. & ROTIN, D. (2011). Deletion of the ubiquitin ligase Nedd4L in lung epithelia causes cystic fibrosis-like disease. *Proc Natl Acad Sci USA,* **108:** 3216-21.

KLEYMAN, T. R., MYERBURG, M. M. & HUGHEY, R. P. (2006). Regulation of ENaCs by proteases: An increasingly complex story. *Kidney Int,* **70:** 1391-2.

KNIGHT, K. K., OLSON, D. R., ZHOU, R. & SNYDER, P. M. (2006). Liddle's syndrome mutations increase Na^+ transport through dual effects on epithelial Na^+ channel surface expression and proteolytic cleavage. *Proc Natl Acad Sci USA,* **103:** 2805-8.

KNIGHT, K. K., WENTZLAFF, D. M. & SNYDER, P. M. (2008). Intracellular sodium regulates proteolytic activation of the epithelial sodium channel. *J Biol Chem,* **283:** 27477-82.

KOBAYASHI, T. & COHEN, P. (1999). Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *Biochem J,* **339:** 319-28.

KOBAYASHI, T., DEAK, M., MORRICE, N. & COHEN, P. (1999). Characterization of the structure and regulation of two novel isoforms of serum- and glucocorticoid-induced protein kinase. *Biochem J,* **344:** 189-197.

KOKAME, K., KATO, H. & MIYATA, T. (1996). Homocysteine-respondent genes in vascular endothelial cells identified by differential display analysis. GRP78/BiP and novel genes. *J Biol Chem,* **271:** 29659-65.

KONSTAS, A. A., SHEARWIN-WHYATT, L. M., FOTIA, A. B., DEGGER, B., RICCARDI, D., COOK, D. I., KORBMACHER, C. & KUMAR, S. (2002). Regulation of the epithelial sodium channel by N4WBP5A, a novel Nedd4/Nedd4-2-interacting protein. *J Biol Chem,* 277**,** 29406-16.

KOSARI, F., SHENG, S. H., LI, J. Q., MAK, D. O. D., FOSKETT, J. K. & KLEYMAN, T. R. (1998). Subunit stoichiometry of the epithelial sodium channel. *J Biol Chemi,* **273:** 13469-74.

KUEHN HS, JUNG MY, BEAVEN MA, METCALFE DD & GILFILLAN AM. (2011). Prostaglandin E2 Activates and Utilizes mTORC2 as a Central Signaling Locus for the Regulation of Mast Cell Chemotaxis and Mediator Release, pp. 391. ASBMB.

KULAKSIZ H, SCHMID A, HÖNSCHEID M, RAMASWAMY A, CETIN Y. 2002. *Proc Natl Acad Sci USA*, **99:** 6796-801.

LAGERCRANTZ, H. & BISTOLETTI, P. (1977). Cathecholamine release in the newborn infant at birth. *Pediatr Res.*, **11:** 889-93.

LANG, F., ARTUNC, F. & VALLON, V. (2009). The physiological impact of the serum and glucocorticoid-inducible kinase SGK1. *Curr Opin Nephrol Hypertens,* **18:** 439-48.

LANG, F. & GÖRLACH, A. (2010). Heterocyclic indazole derivatives as SGK1 inhibitors, WO2008138448. *Expert Opin Ther Pat,* **20:** 129-35.

LAZRAK A, ILES K.E, LIU G., NOAH D.L, NOAH J.W & MATALON S. (2009). Influenza virus M2 protein inhibits epithelial sodium channels by increasing reactive oxygen species, pp.3829. FASEB.

LAZRAK, A. & MATALON, S. (2003). cAMP-induced changes of apical membrane potentials of confluent H441 monolayers. *Am J Physiol Lung Cell Mol Physiol,* **285:** 443-50.

LAZRAK, A., SAMANTA, A., VENETSANOU, K., BARBRY, P. & MATALON, S. 2000. Modification of biophysical properties of lung epithelial Na^+ channels by dexamethasone. *Am J Physiol-Cell Physiol,* **279:** 762-70.

LEE, I. H., DINUDOM, A., SANCHEZ-PEREZ, A., KUMAR, S. & COOK, D. I. (2007). Akt mediates the effect of insulin on epithelial sodium channels by inhibiting Nedd4-2. *J Biol Chem,* **282:** 29866-29873.

LI, T. B. & FOLKESSON, H. G. (2006). RNA interference for alpha-ENaC inhibits rat lung fluid absorption in vivo. *Am J Physiol-Lung Cell Mol Physiol,***290:**649-60.

LIANG, X., BUTTERWORTH, M. B., PETERS, K. W., WALKER, W. H. & FRIZZELL, R. A. (2008). An obligatory heterodimer of 14-3-3beta and 14-3-3epsilon is required for aldosterone regulation of the epithelial sodium channel. *J Biol Chem,* **283:** 27418-25.

LIANG, X., PETERS, K. W., BUTTERWORTH, M. B. & FRIZZELL, R. A. (2006). 14-3-3 isoforms are induced by aldosterone and participate in its regulation of epithelial sodium channels. *J Biol Chem,* **281:** 16323-32.

LIANG, X. B., BUTTERWORTH, M. B., PETERS, K. W. & FRIZZELL, R. A. (2010). AS160 Modulates Aldosterone-stimulated Epithelial Sodium Channel Forward Trafficking. *Mol Biol Cell,* **21:** 2024-33.

LIGGINS G. C., HOWIE, R. N. (1972). A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. *Pediatrics*. **50:** 515-25.

LIN, H. H., ZENTNER, M. D., HO, H. L. L., KIM, K. J. & ANN, D. K. (1999). The gene expression of the amiloride-sensitive epithelial sodium channel alpha-subunit is regulated by antagonistic effects between glucocorticoid hormone and Ras pathways in salivary epithelial cells. *J Biol Chem,* **274:** 21544-54.

LOFFING, J., PIETRI, L., AREGGER, F., BLOCH-FAURE, M., ZIEGLER, U., MENETON, P., ROSSIER, B. C. & KAISSLING, B. (2000). Differential subcellular localization of ENaC subunits in mouse kidney in response to high- and low-Na^+ diets. *Am J Physiol Renal Physiol,* **279:** 252-8.

LOFFING, J., ZECEVIC, M., FÉRAILLE, E., KAISSLING, B., ASHER, C., ROSSIER, B. C., FIRESTONE, G. L., PEARCE, D. & VERREY, F. (2001). Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. *Am J Physiol Renal Physiol,* **280:** 675-82.

LU, M., WANG, J., JONES, K. T., IVES, H. E., FELDMAN, M. E., YAO, L. J., SHOKAT, K. M., ASHRAFI, K. & PEARCE, D. (2010). mTOR complex-2 activates ENaC by phosphorylating SGK1. *J Am Soc Nephrol,* **21:** 811-8.

MA, H. P., CHOU, C. F., WEI, S. P. & EATON, D. C. (2007). Regulation of the epithelial sodium channel by phosphatidylinositides: experiments, implications, and speculations. *Pflugers Arch,* **455:** 169-80.

MACE, O. J. & BAINES, D. L. (2009). Protease activity increases the amiloride-sensitivity of ENaC in human bronchial H441 epithelial cell monolayers. *J Physiol Sci,* **59:** 250.

MACE, O. J., WOOLLHEAD, A. M. & BAINES, D. L. (2008). AICAR activates AMPK and alters PIP_2 association with the epithelial sodium channel ENaC to inhibit Na^+ transport in H441 lung epithelial cells. *J Physiol,* **586:** 4541-57.

MACIAS, M. J., WIESNER, S. & SUDOL, M. (2002). WW and SH3 domains, two different scaffolds to recognize proline-rich ligands. *Febs Letters,* **513:** 30-37.

MALIK, B., YUE, Q., YUE, G., CHEN, X. J., PRICE, S. R., MITCH, W. E. & EATON, D. C. (2005). Role of Nedd4-2 and polyubiquitination in epithelial sodium channel degradation in untransfected renal A6 cells expressing endogenous ENaC subunits. *Am J Physiol Renal Physiol,* **289:**107-16.

MALL, M., BLEICH, M., GREGER, R., SCHREIBER, R. & KUNZELMANN, K. (1998). The amiloride-inhibitable Na^+ conductance is reduced by the cystic fibrosis transmembrane conductance regulator in normal but not in cystic fibrosis airways. *J Clin Invest,* **102:** 15-21.

MALL, M., GRUBB, B. R., HARKEMA, J. R., O'NEAL, W. K. & BOUCHER, R. C. (2004). Increased airway epithelial Na^+ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med,* **10:** 487-93.

MALL, M., HIPPER, A., GREGER, R. & KUNZELMANN, K. (1996). Wild type but not Delta F508 CFTR inhibits Na^+ conductance when coexpressed in Xenopus oocytes. *Febs Letters,* **381:** 47-52.

MALL, M. A., BUTTON, B., JOHANNESSON, B., ZHOU, Z., LIVRAGHI, A., CALDWELL, R. A., SCHUBERT, S. C., SCHULTZ, C., O'NEAL, W. K., PRADERVAND, S., HUMMLER, E., ROSSIER, B. C., GRUBB, B. R. & BOUCHER, R. C. (2010). Airway surface liquid volume regulation determines different airway phenotypes in liddle compared with beta ENaC-overexpressing mice. *J Biol Chem,* **285:** 26945-55.

MANIATIS N.A., CHERNAYA O., SHININ V., MINSHALL R.D. 2012. Caveolins and Lung Function. *Adv Exp Med Biol* **729:** 157-79

MANSLEY, M. K.. (2010). *Hormonal Regulation of Na^+ transport in Murine Collecting Duct Cells.* PhD, University of Dundee.

MANSLEY, M. K. & WILSON, S. M. (2010a). Dysregulation of epithelial Na^+ absorption induced by inhibition of the kinases TORC1 and TORC2. *Br J Pharmacol,* **161:** 1778-92.

MANSLEY, M. K. & WILSON, S. M. (2010b). Effects of nominally selective inhibitors of the kinases PI3K, SGK1 and PKB on the insulin-dependent control of epithelial Na^+ absorption. *Br J Pharmacol,* **161:** 571-88.

MARTINI, F., H., (2004a). The Respiratory System. In *Fundamentals of Anatomy and Physiology*, 6th edn, **pp. 826-873**. Pearson Education Inc, Benjamin Cummings, CA, USA.

MARTINI, F., H., (2004b). The Tissue Level of Organization. In *Fundamentals of Anatomy and Physiology*, 6th edn, **pp. 110-153**. Pearson Education Inc, Benjamin Cummings, CA, USA.

MASON RJ, WILLIAMS MC: Type II alveolar cell: Defender of the alveolus. *Am Rev Respir Dis* 1977, 115:81-91

MASILAMANI, S., KIM, G. H., MITCHELL, C., WADE, J. B. & KNEPPER, M. A. (1999). Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J Clin Invest,* **104:** 19-23.

MASON RJ, WILLIAMS MC (1991). Alveolar type II cells. In: Crystal RG, West JB (eds): The Lung Scientific Foundation. Vol. 1. New York: Raven Press, , p. 235.

MATALON, S. & O'BRODOVICH, H. (1999). Sodium channels in alveolar epithelial cells: Molecular characterization, biophysical properties, and physiological significance. *Ann Rev Physiol,* **61:** 627-61.

MATTHAY, M. A., FOLKESSON, H. G. & CLERICI, C. (2002). Lung epithelial fluid transport and the resolution of pulmonary edema. *Physiol Rev,* **82:** 569-600.

MCDONALD, F. J., PRICE, M. P., SNYDER, P. M. & WELSH, M. J. (1995). Cloning and expression of the beta-subunit and gamma subunit of the human epithelial sodium channel. *Am J Physiol-Cell Physiol,* **268:** 1157-63.

MCDONALD, F. J., WESTERN, A. H., MCNEIL, J. D., THOMAS, B. C., OLSON, D. R. & SNYDER, P. M. (2002). Ubiquitin-protein ligase WWP2 binds to and downregulates the epithelial Na^+ channel. *Am J Physiol-Renal Physiol,* **283:** 431-6.

MCELROY M.C. & KASPER M. (2004). The use of alveolar epithelial type I cell-selective markers to investigate lung injury and repair. *Eur Resp J.* **24:** 664-73

MCNICHOLAS, C. & CANESSA, C. (1997). Diversity of channels generated by different combinations of epithelial sodium channel subunits. *J Gen Physiol***,** 681-692.

MCTAVISH, N., GETTY, J., BURCHELL, A. & WILSON, S. M. (2009). Glucocorticoids can activate the alpha-ENaC gene promoter independently of SGK1. *Biochem J,* **423:** 189-97.

MICK, V. E., ITANI, O. A., LOFTUS, R. W., HUSTED, R. F., SCHMIDT, T. J. & THOMAS, C. P. (2001). The alpha-subunit of the epithelial sodium channel is an aldosterone-induced transcript in mammalian collecting ducts, and this transcriptional response is mediated via distinct cis-elements in the 5 '-flanking region of the gene. *Mol Endocrinol,* **15:** 575-88.

MILLER, A. M., BRESTOFF, J. R., PHELPS, C. B., BERK, E. Z. & REYNOLDS, T. H. (2008). Rapamycin does not improve insulin sensitivity despite elevated mammalian target of rapamycin complex 1 activity in muscles of ob/ob mice. *Am J Physiol-Reg Int Comp Physiol,* **295:** 1431-8.

MOORE, G. E., ITO, E., ULRICH, K. & SANDBERG, A. A. (1966). Culture of human leukemia cells. *Cancer,* **19:** 713-23.

MORA, A., KOMANDER, D., VAN AALTEN, D. M. F. & ALESSI, D. R. (2004). PDK1, the master regulator of AGC kinase signal transduction. *Seminars in Cell Dev Biol,* **15:** 161-170.

MORRIS, R. G. & SCHAFER, J. A. (2002). cAMP increases density of ENaC subunits in the apical membrane of MDCK cells in direct proportion to amiloride-sensitive Na^+ transport. *J Gen Physiol,* **120:** 71-85.

MURRAY, J. T., CAMPBELL, D. G., MORRICE, N., AULD, G. C., SHPIRO, N., MARQUEZ, R., PEGGIE, M., BAIN, J., BLOOMBERG, G. B., GRAHAMMER, F., LANG, F., WULFF, P., KUHL, D. & COHEN, P. (2004). Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3. *Biochem J,* **384:** 477-88.

MURRAY, J. T., CUMMINGS, L. A., BLOOMBERG, G. B. & COHEN, P. (2005). Identification of different specificity requirements between SGK1 and PKB alpha. *Febs Letters,* **579:** 991-4.

MUSLIN, A. J., TANNER, J. W., ALLEN, P. M. & SHAW, A. S. (1996). Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell,* **84:** 889-97.

MUSTAFA, S. B., DIGERONIMO, R. J., PETERSHACK, J. A., ALCORN, J. L. & SEIDNER, S. R. (2004). Postnatal glucocorticoids induce alpha-ENaC formation and regulate glucocorticoid receptors in the preterm rabbit lung. *Am J Physiol-Lung Cell Mol Physiol,* **286:** 73-80.

MUTLU, G. M. & SZNAJDER, J. I. (2005). Mechanisms of pulmonary edema clearance. *Am Physiol-Lung Cell Mol Physiol,* **289:** 685-95.

MYERBURG, M. M., BUTTERWORTH, M. B., MCKENNA, E. E., PETERS, K. W., FRIZZELL, R. A., KLEYMAN, T. R. & PILEWSKI, J. M. (2006). Airway surface liquid volume regulates ENaC by altering the serine protease-protease inhibitor balance - A mechanism for sodium hyperabsorption in cystic fibrosis. *J Biol Chem,* **281:** 27942-9.

MYERBURG M.M, HARVEY P.R, HEIDRICH E.M, PILEWSKI J.M & BUTTERWORTH M.B. (2010). Acute regulation of the epithelial sodium channel in airway epithelia by proteases and trafficking, pp. 712.

NAGAKI, K., YAMAMURA, H., SHIMADA, S., SAITO, T., HISANAGA, S., TAOKA, M., ISOBE, T. & ICHIMURA, T. (2006). 14-3-3 Mediates phosphorylation-dependent inhibition of the interaction between the ubiquitin E3 ligase Nedd4-2 and epithelial Na^+ channels. *Biochem,* **45:** 6733-40.

NAKAMURA, K., STOKES, J. B. & MCCRAY, P. B. (2002). Endogenous and exogenous glucocorticoid regulation of ENaC mRNA expression in developing kidney and lung. *Am J Physiol-Cell Physiol,* **283:** 762-72.

NARAY-FEJES-TOTH, A., CANESSA, C., CLEAVELAND, E. S., ALDRICH, G. & FEJES-TOTH, G. (1999). SGK is an aldosterone-induced kinase in the renal collecting duct - Effects on epithelial Na^+ channels. *J Biol Chem,* **274:**16973-8.

NASCIMENTO, E. B. M., SNEL, M., GUIGAS, B., VAN DER ZON, G. C. M., KRIEK, J., MAASSEN, J. A., JAZET, I. M., DIAMANT, M. & OUWENS, D. M. (2010). Phosphorylation of PRAS40 on Thr^{246} by PKB/AKT facilitates efficient phosphorylation of Ser183 by mTORC1. *Cell Signal,* **22:** 961-7.

NIELSEN S, KING LS, CHRISTENSEN BM & AGRE P. (1997). Aquaporins in complex tissues.II. Subcellular distribution in respiratory and glandular tissues of rat, pp. C1549. *Am Physiological Soc.*

NORMAND I.C.S, OLVER R.E, REYNOLDS EO.R, STRANG L.B & WELCH K. (1971). Permeability of lung capillaries and alveoli to non-electrolytes in the foetal lamb. With an Appendix, pp. 303. *Physiol Soc.*

O'BRODOVICH, H. (2004). Deficient Na^+ channel expression in newborn respiratory distress syndrome. *Pediatr pulmonol. Suppl* **26:** 141-2.

O'BRODOVICH, H. M. (1996). Immature epithelial Na^+ channel expression is one of the pathogenetic mechanisms leading to human neonatal respiratory distress syndrome. *Proc Assoc Am Physicians,* **108:** 345-55.

O'BRODOVICH, H. M. HANNAM, V., SEEAR, M. & MULLEN, J.B. (1990). Amiloride impairs lung water clearance in newborn guine pigs. *J Appl Physiol,* **68:** 1758-62.

O'ROHILLY R., MULLER F., CARPENTER S., SWENSON R. (2004) Basic Human Anatomy: A regional Study of Human Structure. Chapter 30: Blood vessels, lymphatic drainage and nerves of the abdomen. Dartmouth Medical School, USA

O'BRODOVICH, H., YANG, P., GANDHI, S. & OTULAKOWSKI, G. (2008). Amiloride-insensitive Na^+ and fluid absorption in the mammalian distal lung. *Am J Physiol-Lung Cell Mol Physiol,* **294:** 401-8.

OLVER, R. E., RAMSDEN, C. A., STRANG, L. B. & WALTERS, D. V. (1986). The role of amiloride-blockable sodium-transport in adrenaline-induced lung liquid reabsorption in the fetal lamb. *J Physiol (Lond),* **376:** 321-40.

OLVER, R. E., RAMSDEN, C. A. & WALTERS, D. V. (1987). The effect of dibutyryl-cyclic AMP on lung luquid volume flow across the pulmonary epithelium of the fetal sheep. *J Physiol (Lond),* **391:** 61.

OLVER, R. E. & STRANG, L. B. (1974). Ion fluxes across pulmonary epithelium and secretion of lung liquid in fetal lamb. *J Physiol (Lond),* **241:** 327-57.

OLVER, R. E., WALTERS, D. V. & WILSON, S. M. (2004). Development regulation of lung liquid transport. *Ann Rev Physiol,* **66:** 77-101.

OSHIRO N, TAKAHASHI R, YOSHINO K, TANIMURA K, NAKASHIMA A, EGUCHI S, MIYAMOTO T, HARA K, TAKEHANA K & AVRUCH J. (2007). The proline-rich Akt substrate of 40 kDa (PRAS40) is a physiological substrate of mammalian target of rapamycin complex 1, pp. 20329. ASBMB.

OTULAKOWSKI, G., DUAN, W., GANDHI, S. & O'BRODOVICH, H. (2007). Steroid and oxygen effects on elF4F complex, mTOR, and ENaC translation in fetal lung epithelia. *Am J Respir Cell Mol Biol,* **37:** 457-66.

OTULAKOWSKI, G., RAFII, B., BREMNER, H. R. & O'BRODOVICH, H. (1999). Structure and hormone responsiveness of the gene encoding the alpha-subunit of the rat amiloride-sensitive epithelial sodium channel. *Am Respir Cell Mol Biol,* **20:** 1028-40.

OTULAKOWSKI, G., RAFII, B., HARRIS, M. & O'BRODOVICH, H. (2006). Oxygen and glucocorticoids modulate alpha ENaC mRNA translation in fetal distal lung epithelium. *Am Respir Cell Mol Biol,* **34:** 204-12.

PALMER, L. G. (1992). Epithelial Na^+ Channels – Function and Diversity. *Ann Rev Physiol,* **54:** 51-66.

PARK, J., LEONG, M. L. L., BUSE, P., MAIYAR, A. C., FIRESTONE, G. L. & HEMMINGS, B. A. (1999). Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. *Embo Journal,* **18:** 3024-33.

PASSERO CJ, MUELLER GM, RONDON-BERRIOS H, TOFOVIC SP, HUGHEY RP & KLEYMAN TR. (2008). Plasmin activates epithelial Na^+ channels by cleaving the subunit, pp. 36586. ASBMB.

PAUNESCU, T. G., BLAZER-YOST, B. L., VLAHOS, C. J. & HELMAN, S. I. (2000). LY-294002-inhibitable PI 3-kinase and regulation of baseline rates of Na^+ transport in A6 epithelia. *Am Physiol Cell Physiol,* **279:** 236-47.

PEARCE, D. (2001). The role of SGK1 in hormone-regulated sodium transport. *Trends Endocrinol Metab,* **12:** 341-7.

PEARCE, D. & KLEYMAN, T. R. (2007). Salt, sodium channels, and SGK1. *J Clin Invest,* **117:** 592-5.

PEARCE, L. R., KOMANDER, D. & ALESSI, D. R. (2010). The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol,* **11:** 9-22.

PERROTTI, N., HE, R. A., PHILLIPS, S. A., HAFT, C. R. & TAYLOR, S. I. (2001). Activation of serum- and glucocorticoid-induced protein kinase (Sgk) by cyclic AMP and insulin. *J Biol Chem,* **276:** 9406-12.

PLANES, C., BLOT-CHABAUD, M., COUETTE, S., MATTHAY, A. A. & CLERICI, C. (2002). The hypoxia-induced decrease in sodium transport in alveolar type II cells is mediated by decreased delivery of ENaC to the apical membrane. *J Biol Chem,* **277:** 47318-24.

PLANÈS, C., LEYVRAZ, C., UCHIDA, T., ANGELOVA, M. A., VUAGNIAUX, G., HUMMLER, E., MATTHAY, M., CLERICI, C. & ROSSIER, B. (2005). In vitro and in vivo regulation of transepithelial lung alveolar sodium transport by serine proteases. *Am J Physiol Lung Cell Mol Physiol,* **288:** 1099-109.

PLANÈS C, RANDRIANARISON N.H, CHARLES R.P, FRATESCHI S, CLUZEAUD F, VUAGNIAUX G, SOLER P, CLERICI C, ROSSIER B.C & HUMMLER E. (2009). ENaC mediated alveolar fluid clearance and lung fluid balance depend on the channel activating protease 1, pp. 26-37. Wiley Online Library.

PLANT, P. J., YEGER, H., STAUB, O., HOWARD, P. & ROTIN, D. (1997). The C2 domain of the ubiquitin protein ligase Nedd4 mediates $Ca2^{+}$-dependent plasma membrane localization. *J Biol Chem,* **272:** 32329-36.

POCHYNYUK, O., TONG, Q., STARUSCHENKO, A., MEDINA, J. & STOCKAND, J. D. (2007a). Molecular determinants of PI(4,5)P-2 and PI(3,4,5)P-3 regulation of the epithelial Na^{+} channel. *J Gen Physiol*., 130: 399-413.

POCHYNYUK, O., TONG, Q. S., STARUSCHENKO, A. & STOCKAND, J. D. (2007b). Binding and direct activation of the epithelial Na^{+} channel (ENaC) by phosphatidylinositides. *J Physiol (Lond),* **580:** 365-72.

POTTER, E.L., BOHLENDER, G.P. (1941). Intrauterine respiration in relation to development of the fetal lung. *Am. J. Obstet. Gynecol.* 42, 14–22.

PRINCE, L. S. & WELSH, M. J. (1998). Cell surface expression and biosynthesis of epithelial Na^{+} channels. *Biochem J,* **336:** 705-10.

PROUD, C. G. (2007). Signalling to translation: how signal transduction pathways control the protein synthetic machinery. *Biochem J,* **403:** 217-34.

RAIKWAR, N. S. & THOMAS, C. P. (2008). Nedd4-2 isoforms ubiquitinate individual epithelial sodium channel subunits and reduce surface expression and function of the epithelial sodium channel. *Am Physiol Renal Physiol,* **294:** 1157-65.

RAMEH, L.E. & CANTELY, L.C. (1999). The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol Chem*, **274:** 8347-50.

RAMMINGER, S. J., BAINES, D. L., OLVER, R. E. & WILSON S. M. 2000. The effects of PO_2 upon transpithelial ion transport in fetal rat distal lung epithelial cells. *J Physiol*. 524, 539-547.

RAMMINGER, S. J., OLVER, R. E. & WILSON, S. M. (2002). Hormonal regulation of apical membrane Na^{+} conductance in rat fetal distal lung epithelial cells. *J Physiol,* **544:** 567-77.

RAMMINGER, S. J., RICHARD, K., INGLIS, S. K., LAND, S. C., OLVER, R. E. & WILSON, S. M. 2004. A regulated apical Na^{+} conductance in dexamethasone-treated H441 airway epithelial cells. *Am Physiol-Lung Cell Mol Physiol,* **287:** 411-9.

RANDRIANARISON, N., CLERICI, C., FERREIRA, C., FONTAYNE, A., PRADERVAND, S., FOWLER-JAEGER, N., HUMMLER, E., ROSSIER, B. C. & PLANES, C. (2008). Low expression of the beta-ENaC subunit impairs lung fluid clearance in the mouse. *Am Physiol-Lung Cell Mol Physiol,* **294:** 409-16.

RAYNAUD, F. I., ECCLES, S., CLARKE, P. A., HAYES, A., NUTLEY, B., ALIX, S., HENLEY, A., DI-STEFANO, F., AHMAD, Z., GUILLARD, S., BJERKE, L. M., KELLAND, L., VALENTI, M., PATTERSON, L., GOWAN, S., BRANDON, A. D. H., HAYAKAWA, M., KAIZAWA, H., KOIZUMI, T., OHISHI, T., PATEL, S., SAGHIR, N., PARKER, P., WATERFIELD, M. & WORKMAN, P. (2007). Pharmacologic characterization of a potent inhibitor of class I phosphatidylinositide 3-kinases. *Cancer Res,* **67:** 5840-50.

RAZANI B, ENGELMAN JA,WANG XB, SCHUBERTW, ZHANG XL, MARKS CB, MACALUSO F, RUSSELL RG, LI M, PESTELL RG, DI VIZIO D, HOU H JR, KNEITZ B, LAGAUD G, CHRIST GJ, EDELMANN W, LISANTI MP (2001) Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J Biol Chem* **276(41)**:38121–38138

RECORD, R. D., FROELICH, L. L., VLAHOS, C. J. & BLAZER-YOST, B. L. (1998). Phosphatidylinositol 3-kinase activation is required for insulin-stimulated sodium transport in A6 cells. *Am Physiol-Endocrinol Met,* **274:** 611-7.

REISENAUER M.R., WANG S.W., XIA Y., ZHANG W. (2010) Dot1A contains three nuclear localisation signals and regulates the epithelial Na+ Channel (ENaC) at multiple levels. *Am J Physiol Renal Physiol.* 2010 Jul;299(1):F63-76.

REXHEPAJ, R., ARTUNC, F., GRAHAMMER, F., NASIR, O., SANDU, C., FRIEDRICH, B., KUHL, D. & LANG, F. (2006). SGK1 is not required for regulation of colonic ENaC activity. *Pflugers Arch-Eur J Physiol,* **453:** 97-105.

RICCARDI C. (2010). GILZ (glucocorticoid-induced leucine zipper), a mediator of the anti-inflammatory and immunosuppressive activity of glucocorticoids, pp. 53.

ROSSIER, B. C. & STUTTS, M. J. (2009). Activation of the Epithelial Sodium Channel (ENaC) by Serine Proteases. *Ann Rev Physiol,* **71:** 361-79.

ROTIN, D., KANELIS, V. & SCHILD, L. (2001). Trafficking and cell surface stability of ENaC. *Am J Physiol Renal Physiol,* **281:** 391-9.

ROTIN, D., STAUB, O. & HAGUENAUER-TSAPIS, R. (2000). Ubiquitination and endocytosis of plasma membrane proteins: Role of Nedd4/Rsp5p family of ubiquitin-protein ligases. *J Membr Biol,* **176:** 1-17.

RUFFIEUX-DAIDIÉ, D., POIROT, O., BOULKROUN, S., VERREY, F., KELLENBERGER, S. & STAUB, O. (2008). Deubiquitylation regulates activation and proteolytic cleavage of ENaC. *J Am Soc Nephrol,* **19:** 2170-80.

SARBASSOV D. D., ALI S.M. KIM D.H. GUERTIN D.D., LATEK R.R., ERDJUMENT-BROMAGE H., TEMPST P., SABATINI D.M. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol.* **14 (14):** 1296-302.

SARBASSOV D.D, ALI S.M, SENGUPTA S, SHEEN J.H, HSU P.P, BAGLEY A.F, MARKHARD A.L & SABATINI D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB, pp. 159-168. Elsevier.

SARBASSOV, D. D., GUERTIN, D. A., ALI, S. M. & SABATINI, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science,* **307:** 1098-101.

SAYEGH, R., AUERBACH, S. D., LI, X., LOFTUS, R. W., HUSTED, R. F., STOKES, J. B. & THOMAS, C. P. (1999). Glucocorticoid induction of epithelial sodium channel expression in lung and renal epithelia occurs via trans-activation of a hormone response element in the 5 '-flanking region of the human epithelial sodium channel alpha subunit gene. *J Biol Chem,* **274:** 12431-7.

SCHNEEBERGER E.E.(1991) Airway and alveolar epithelial cell junctions. In: Crystal RG, West JB (eds): The Lung Scientific Foundation. Vol. 1. New York: Raven Press, p. 205.

SCHILD, L., LU, Y., GAUTSCHI, I., SCHNEEBERGER, E., LIFTON, R. P. & ROSSIER, B. C. (1996). Identification of a PY motif in the epithelial Na channel subunits as a target sequence for mutations causing channel activation found in Liddle syndrome. *EMBO J,* **15:** 2381-7.

SCHILD, L., SCHNEEBERGER, E., GAUTSCHI, I. & FIRSOV, D. (1997). Identification of amino acid residues in the alpha, beta, and gamma subunits of the epithelial sodium channel (ENaC) involved in amiloride block and ion permeation. *J Gen Physiol,* **109:** 15-26.

SHANKS A, GROSS G, SHIM T, ALLSWORTH J, SADOVSKY Y, BILDIRICI I. Administration of steroids after 34 weeks of gestation enhances fetal lung maturity profiles. *Am J Obstet Gynecol.* 2010 Jul;203(1):47.e1-5. Epub 2010 May 15.

SHENG, S., CARATTINO, M. D., BRUNS, J. B., HUGHEY, R. P. & KLEYMAN, T. R. (2006). Furin cleavage activates the epithelial Na^+ channel by relieving Na^+ self-inhibition. *Am Physiol-Renal Physiol,* **290:** 1488-96.

SHERK, A. B., FRIGO, D. E., SCHNACKENBERG, C. G., BRAY, J. D., LAPING, N. J., TRIZNA, W., HAMMOND, M., PATTERSON, J. R., THOMPSON, S. K., KAZMIN, D., NORRIS, J. D. & MCDONNELL, D. P. (2008). Development of a small-molecule serum- and glucocorticoid-regulated kinase-1 antagonist and its evaluation as a prostate cancer therapeutic. *Cancer Res,* **68:** 7475-83.

SHI, P. P., CAO, X. R., SWEEZER, E. M., KINNEY, T. S., WILLIAMS, N. R., HUSTED, R. F., NAIR, R., WEISS, R. M., WILLIAMSON, R. A., SIGMUND, C. D., SNYDER, P. M., STAUB, O., STOKES, J. B. & YANG, B. (2008). Salt-sensitive hypertension and cardiac hypertrophy in mice deficient in the ubiquitin ligase Nedd4-2. *Am Physiol-Renal Physiol,* **295:** 462-70.

SHIMKETS, R. A., WARNOCK, D. G., BOSITIS, C. M., NELSONWILLIAMS, C., HANSSON, J. H., SCHAMBELAN, M., GILL, J. R., ULICK, S., MILORA, R. V., FINDLING, J. W., CANESSA, C. M., ROSSIER, B. C. & LIFTON, R. P. 1994. Liddle's Syndrome - heritable human hypertension caused by mutations in the beta-subunit of the epithelial sodium channel. *Cell,* **79:** 407-14.

SHLYONSKY, V., GOOLAERTS, A., VAN BENEDEN, R. & SARIBAN-SOHRABY, S. (2005). Differentiation of epithelial Na^+ channel function. An in vitro model. *J Biol Chem,* **280:** 24181-7.

SILVA, P., STOFF, J., FIELD, M., FINE, L., FORREST, J., N., & EPSTEIN, F., H. (1977). Mechanism of active chloride secretion by shark rectal gland: role of Na^+-K^+-ATPase in chloride transport. *Am J Physiol Renal Physiol,* **233:** 298-306.

SMITH, D. E., OTULAKOWSKI, G., YEGER, H., POST, M., CUTZ, E. & O'BRODOVICH, H. M. (2000). Epithelial Na^+ channel (ENaC) expression in the developing normal and abnormal human perinatal lung. *Am J Respir Crit Care Med,* **161:** 1322-31.

SMITH, P. R. & BENOS, D. J. (1991). Epithelial Na^+ Channels. *Ann Rev Physiol,* **53:** 509-30.

SNYDER, P. M. (2000). Liddle's syndrome mutations disrupt cAMP-mediated translocation of the epithelial Na^+ channel to the cell surface. *J Clin Invest* **105:** 45-53.

SNYDER, P. M. (2002). The epithelial Na^+ channel: Cell surface insertion and retrieval in Na^+ homeostasis and hypertension. *Endocr Rev,* **23:** 258-75.

SNYDER, P. M. (2009). Down-Regulating Destruction: Phosphorylation Regulates the E3 Ubiquitin Ligase Nedd4-2. *Sci Signal,* **2:** 41.

SNYDER, P. M., CHENG C , PRINCE L. S. , ROGERS J. C. , WELSH M. J. (1998). Electrophysiological and biochemical evidence that DEG/ENaC cation channels are composed of nine subunits. *J Biol Chem*, **273:** 681-4.

SNYDER P.M., OLSON D.R., MCDONALD F.J., BUCHER D.B. (2001). Multiple WW domains, but not the C2 domain, are required for inhibition of the epithelial Na^+ channel by human Nedd4. *J Biol Chem*, **276:** 28321-6.

SNYDER, P. M., OLSON, D. R., KABRA, R., ZHOU, R. &STEINES, J.C. (2004a). cAMP and serum glucocorticoid-inducible kinase (SGK) regulate the epithelial Na^+ channel through convergent phosphorylation of Nedd4-2. *J Biol Chem*, **279:** 45753-8

SNYDER, P. M., OLSON, D. R. & THOMAS, B. C. (2002). Serum and glucocorticoid-regulated kinase modulates Nedd4-2-mediated inhibition of the epithelial Na^+ channel. *J Biol Chem,* **277:** 5-8.

SNYDER, P. M., STEINES, J. C. & OLSON, D. R. (2004b). Relative contribution of Nedd4 and Nedd4-2 to ENaC regulation in epithelia determined by RNA interference. *J Biol Chem,* **279:** 5042-6.

SONG, J., HU, X. Q., RIAZI, S., TIWARI, S., WADE, J. B. & ECELBARGER, C. A. (2006). Regulation of blood pressure, the epithelial sodium channel (ENaC), and other key renal sodium transporters by chronic insulin infusion in rats. *Am J Physiol-Renal Physiol,* **290:** 1055-64.

STARUSCHENKO, A., ADAMS, E., BOOTH, R. E. & STOCKAND, J. D. (2005). Epithelial Na^+ channel subunit stoichiometry. *Biophys J,* **88:** 3966-75.

STAUB, O., ABRIEL, H., PLANT, P., ISHIKAWA, T., KANELIS, V., SALEKI, R., HORISBERGER, J. D., SCHILD, L. & ROTIN, D. (2000). Regulation of the epithelial Na^+ channel by Nedd4 and ubiquitination. *Kidney Int,* **57:** 809-15.

STAUB, O., DHO, S., HENRY, P., CORREA, J., ISHIKAWA, T., MCGLADE, J. & ROTIN, D. (1996). WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na^+ channel deleted in Liddle's syndrome. *EMBO J,* **15:** 2371-80.

STAUB, O., GAUTSCHI, I., ISHIKAWA, T., BREITSCHOPF, K., CIECHANOVER, A., SCHILD, L. & ROTIN, D. (1997a). Regulation of stability and function of the epithelial Na^+ channel (ENaC) by ubiquitination. *EMBO J,* **16:** 6325-36.

STAUB, O. & VERREY, F. (2005). Impact of Nedd4 proteins and serum and glucocorticoid-induced kinases on epithelial Na^+ transport in the distal nephron. *J Am Soc Nephrol,* **16:** 3167-74.

STAUB, O., YEGER, H., PLANT, P. J., KIM, H., ERNST, S. A. & ROTIN, D. (1997). Immunolocalization of the ubiquitin-protein ligase Nedd4 in tissues expressing the epithelial Na^+ channel (ENaC). *Am J Physiol,* **272:** 1871-80.

TALBOT, C. L., BOSWORTH, D. G., BRILEY, E. L., FENSTERMACHER, D. A., BOUCHER, R. C., GABRIEL, S. E. & BARKER, P. M. (1999). Quantitation and localization of ENaC subunit expression in fetal, newborn, and adult mouse lung. *Am Respir Cell Mol Biol,* **20:** 398-406.

TAN C.D, SELVANATHAR I.A, BAINES D.L. (2011). Cleavage of endogenous gamma ENaC and elevated abundance of alpha ENaC are associated with increased Na+ transport in response to apical fluid volume expansion in human H441 airway epithelial cells. *Pflügers Arch.* 462, 431-441.

TARRAN, R. (2004). Regulation of airway surface liquid volume and mucus transport by active ion transport. *Proc Am Thorac Soc,* **1:** 42-6.

TCHEPICHEV, S., UEDA, J., CANESSA, C., ROSSIER, B. C. & OBRODOVICH, H. (1995). Lung epithelial Na^+ channel subnits are differentially regulated during development and by steroids. *Am J Physiol-Cell Physiol,* **269:** 805-12.

THOM, J., PERKS, A.M. (1990). The effects of furosemide and bumetanide on lung liquid production by *in vitro* lungs from fetal guinea pigs. Can. *J. Physiol. Pharmacol.* 68, 1131–1135.

THOMAS, C. P., CAMPBELL, J. R., WRIGHT, P. J. & HUSTED, R. F. (2004). cAMP-stimulated Na^+ transport in H441 distal lung epithelial cells: role of PKA, phosphatidylinositol 3-kinase, and sgk1. *Am J Physiol-Lung Cell Mol Physiol,* **287:** 843-51.

THOMAS, C. P., LOFTUS, R. W., LIU, K. Z. & ITANI, O. A. (2002). Genomic organization of the 5 ' end of human beta-ENaC and preliminary characterization of its promoter. *Am J Physiol-Renal Physiol,* **282:** 898-909.

THOMAS C.P. & ITANI O.A. (2004). New insights into epithelial sodium channel function in the kidney: site of action, regulation by ubiquitin ligases, serum- and glucocorticoid-inducible kinase and proteolysis. *Curr Opin Nephrol Hypertens,* **13:** 541-8.

THOREEN, C. C., KANG, S. A., CHANG, J. W., LIU, Q., ZHANG, J., GAO, Y., REICHLING, L. J., SIM, T., SABATINI, D. M. & GRAY, N. S. (2009). An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J Biol Chem,* **284:** 8023-32.

THOREEN, C. C. & SABATINI, D. M. (2009). Rapamycin inhibits mTORC1, but not completely. *Autophagy,* **5:** 725-6.

TIWARI, S., NORDQUIST, L., HALAGAPPA, V. K. M. & ECELBARGER, C. A. (2007). Trafficking of ENaC subunits in response to acute insulin in mouse kidney. *Am J Physiol-Renal Physiol,* **293:** 178-85.

TOKUNAGA C, YOSHINO K, YONEZAWA K (2004). mTOR integrates amino acid- and energy-sensing pathways. *Biochem Biophys Res Commun* **313** (2): 443–6.

VALLET, V., CHRAIBI, A., GAEGGELER, H. P., HORISBERGER, J. D. & ROSSIER, B. C. (1997). An epithelial serine protease activates the amiloride-sensitive sodium channel. *Nature,* **389:** 607-10.

VALLET V, PFISTER C, LOFFING J & ROSSIER BC. (2002). Cell-surface expression of the channel activating protease xCAP-1 is required for activation of ENaC in the Xenopus oocyte, pp. 588. *Am Soc Nephrol.*

VASQUEZ, M. M., CASTRO, R., SEIDNER, S. R., HENSON, B. M., ASHTON, D. J. & MUSTAFA, S. B. (2008). Induction of Serum- and Glucocorticoid-Induced Kinase-1 (SGK1) by cAMP Regulates Increases in alpha-ENaC. *J Cell Physiol,* **217:** 632-42.

VENKATESH, V. C. & KATZBERG, H. D. (1997). Glucocorticoid regulation of epithelial sodium channel genes in human fetal lung. *Am J Physiol-Lung Cell Mol Physiol,* **273:** 227-33.

VERGHESE GM, TONG ZY, BHAGWANDIN V & CAUGHEY GH. (2004). Mouse prostasin genestructure, promoter analysis, and restricted expression in lung and kidney, pp. 519-529.*Am Thor Soc.*

VIEL, M., LEROY, C., HUBERT, D., FAJAC, I. & BIENVENU, T. (2008). ENaC beta and gamma genes as modifier genes in cystic fibrosis. *J Cyst Fibros,* **7:** 23-9.

VON NEERGAARD K**.** (1929) Neue Auffassungen uber einen Grundbegriff der Atemmechanik. Die Retraktionskraft der Lunge, abhangig von der Oberflachenspannung in den Alveolen. *Z Gesamte Exp Med* 66: 373–394.

VUAGNIAUX, G., VALLET, V., JAEGER, N. F., PFISTER, C., BENS, M., FARMAN, N., COURTOIS-COUTRY, N., VANDEWALLE, A., ROSSIER, B. C. & HUMMLER, E. (2000). Activation of the amiloride-sensitive epithelial sodium channel by the serine protease mCAP1 expressed in a mouse cortical collecting duct cell line. *J Am Soc Nephrol,* **11:** 828-34.

WAGNER, C. A., OTT, M., KLINGEL, K., BECK, S., MELZIG, J., FRIEDRICH, B., WILD, K. N., BROER, S., MOSCHEN, I., ALBERS, A., WALDEGGER, S., TUMMLER, B., EGAN, M. E., GEIBEL, J. P., KANDOLF, R. & LANG, F. (2001). Effects of the serine/threonine kinase SGK1 on the epithelial Na^+ channel (ENaC) and CFTR: Implications for cystic fibrosis. *Cell Physiol Biochem,* **11:** 209-218.

WALDEGGER S, BARTH P, RABER G, LANG F (1997). Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during anisotonic and isotonic alterations of cell volume. *Proc Natl Acad Sci USA.* 4 (9): 4440–5.

WALDMANN, R., CHAMPIGNY, G., BASSILANA, F., VOILLEY, N. & LAZDUNSKI, M. (1995). Molecular-cloning and functional expression of a novel amiloride sensitive Na^+ channel. *J Biol Chem,* **270:** 27411-14.

WALTER, D., V. & OLVER, R., E. (1978). The Role of catecholamines in lung liquid absorption at birth. *Pediatr Res*., **12:** 239-42.

WALTERS, D. V., RAMSDEN, C. A. & OLVER, R. E. (1990). Dibutyryl cAMP induces a gestation-dependent absorption of fetal lung liquid. *J Appl Physiol,* **68:** 2054-9.

WANG, J., BARBRY, P., MAIYAR, A. C., ROZANSKY, D. J., BHARGAVA, A., LEONG, M., FIRESTONE, G. L. & PEARCE, D. (2001). SGK integrates insulin and mineralocorticoid regulation of epithelial sodium transport. *Am J Physiol-Renal Physiol,* **280:** 303-13.

WANG, J., KNIGHT, Z. A., FIEDLER, D., WILLIAMS, O., SHOKAT, K. M. & PEARCE, D. (2008). Activity of the p110-alpha subunit of phosphatidylinositol-3-kinase is required for activation of epithelial sodium transport. *Am J Physiol Renal Physiol,* **295:** 843-50.

WANG S., SINGH R.D., GODIN L., PAGANO R.E. HUBMAYR R.D. 2011. Endocytivc response of type I alveolar epithelial cells to hypertonic stress. *Am Lung Physiol* **300 (4):** 560-8.

WATANABE, S., MATSUSHITA, K., STOKES, J. B. & MCCRAY, P. B. (1998). Developmental regulation of epithelial sodium channel subunit mRNA expression in rat colon and lung. *Am J Physiol,* **275:** 1227-35.

WATT G. B. (2011). *The role of Serum and Glucocorticoid-kinase in the hormonal control of sodium transport in pulmonary epithelia.* Ph.D, University of Dundee.

WATT G. B., ISMAIL, N. A. S., CABALLERO, A. G., LAND, S. C., WILSON, S. M. (2012). Glucocorticoid-induced activation of epithelial Na^+ channels in human airway epithelial cells: effects of inhibiting the kinases PI3K, TORC2 and SGK1. *Brit J Pharmacol* **166(4):**1272-89.

WEIBEL E.R. (1963) Morphometry of the Human Lung. Heidelberg: Springer, p. 111.

WEBSTER M. K., GOYA L., GE Y., MAIYAR A. C., FIRESTONE G. L.(1993). Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Mol Cell Biol.,* **13:** 2031-40.

WEDAMAN, K.P., REINKE A., ANDERSON, S., YATES J., MCCAFFERY M., POWERS T. (2003) TOR kinases are in distinct membrane-associated protein complexes in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 14, 1204–1220

WELSH, M., J., WIDDICOMBE, J., H. & NADEL, J., A. (1980). Fluid Transport across the canine tracheal epithelium. *J Appl Physiol*. **49:** 905-9.

WEST, J. (2000). Chapter 5: Ventilation-Perfusion Relationships. In *Respiratory Physiology The Essentials*, 6th Edition, PJK. Lippincott Williams & Wilkins, Baltimore.

WIEMUTH, D., LOTT, J. S., LY, K., KE, Y., TEESDALE-SPITTLE, P., SNYDER, P. M. & MCDONALD, F. J. (2010). Interaction of serum- and glucocorticoid regulated kinase 1 (SGK1) with the WW-domains of Nedd4-2 is required for epithelial sodium channel regulation. *PLoS One,* **5:** e12163.

WILKINSON, W. J., BENJAMIN, A. R., DE PROOST, I., OROGO-WENN, M. C., YAMAZAKI, Y., STAUB, O., MORITA, T., ADRIAENSEN, D., RICCARDI, D., WALTERS, D. V. & KEMP, P. J. (2011). Alveolar epithelial CNGA1 channels mediate cGMP-stimulated, amiloride-insensitive, lung liquid absorption. *Pflugers Arch,* **462:** 267-79.

WOOLLHEAD, A. M. & BAINES, D. L. (2006). Forskolin-induced cell shrinkage and apical translocation of functional enhanced green fluorescent protein-human alpha ENaC in H441 lung epithelial cell monolayers. *J Biol Chem,* **281:** 5158-68.

WULFF, P., VALLON, V., HUANG, D. Y., VÖLKL, H., YU, F., RICHTER, K., JANSEN, M., SCHLÜNZ, M., KLINGEL, K., LOFFING, J., KAUSELMANN, G., BÖSL, M. R., LANG, F. & KUHL, D. 2002. Impaired renal $Na(^{+})$ retention in the sgk1-knockout mouse. *J Clin Invest,* **110:** 1263-8.

WULLSCHLEGER, S., LOEWITH, R. & HALL, M. N. (2006). TOR signaling in growth and metabolism. *Cell,* **124:** 471-84.

XU, H. & CHU, S. (2007). ENaC alpha-subunit variants are expressed in lung epithelial cells and are suppressed by oxidative stress. *Am J Physiol-Lung Cell Mol Physiol,* **293:** 1454-62.

YANG J, CRON P, THOMPSON V, GOOD VM, HESS D, HEMMINGS BA & BARFORD D. (2002). Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation, pp. 1227-1240. Elsevier.

YANG L.M, RINKE R. & KORBMACHER C. (2006). Stimulation of the epithelial sodium channel (ENaC) by cAMP involves putative ERK phosphorylation sites in the C termini of the channel's α-and β-subunit, pp. 9859. ASBMB.

YOO D, KIM BY, CAMPO C, NANCE L, KING A, MAOUYO D, WELLING PA (2003) Cell surface expression of the ROMK (Kir1.1) channel is regulated by the aldosterone-induced kinase, SGK-1, and protein kinase A. *J Biol Chem* 278:23066–23075.

YU, L., HELMS, M. N., YUE, Q. & EATON, D. C. (2008). Single-channel analysis of functional epithelial sodium channel (ENaC) stability at the apical membrane of A6 distal kidney cells. *Am J Physiol Renal Physiol,* **295:** 1519-27.

YUE G, RUSSELL WJ, BENOS DJ, JACKSON RM, OLMAN MA & MATALON S. (1995). Increased expression and activity of sodium channels in alveolar type II cells of hyperoxic rats, *Nat Acad Sciences* pp. 8418.

ZELTNER T.B. & BURRI P.H.: The postnatal development and growth of the human lung. II. Morphology. *Respir Physiol* 67:269, 1987.

ZHANG, W. & WANG, D. X. (2010). Study of regulatory effect of insulin on expression of epithelial sodium channel alpha subunit in A549 cells. *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue*, **22:** 385-8.

ZHANG W, XIA X, REISENAUER M.R, RIEG T., LANG F., KUHL D., VALLON V., KONE B.C. (2007). Aldosterone-induced Sgk1 relieves Dot1a-Af9-mediated transcriptional repression of epithelial Na^+ channel alpha. *J Clin Invest*, **117:** 773-83.

ZHOU, R. F., PATEL, S. V. & SNYDER, P. M. (2007). Nedd4-2 catalyzes ubiquitination and degradation of cell surface ENaC. *J Biol Chem*, **282:** 20207-12.

ZHOU, R. F. & SNYDER, P. M. (2005). Nedd4-2 phosphorylation induces serum and glucocorticoid-regulated kinase (SGK) ubiquitination and degradation. *J Biol Chem*, **280:** 4518-23.

ZHOU, Z., DUERR, J., JOHANNESSON, B., SCHUBERT, S. C., TREIS, D., HARM, M., GRAEBER, S. Y., DALPKE, A., SCHULTZ, C. & MALL, M. A. (2011). The ENaC-overexpressing mouse as a model of cystic fibrosis lung disease. *J Cyst Fibros*, **10 Suppl 2:** 172-82.

ZHOU, Z., TREIS, D., SCHUBERT, S. C., HARM, M., SCHATTERNY, J., HIRTZ, S., DUERR, J., BOUCHER, R. C. & MALL, M. A. (2008). Preventive but not late amiloride therapy reduces morbidity and mortality of lung disease in betaENaC-overexpressing mice. *Am J Respir Crit Care Med*, **178:** 1245-56.

ZOOROB R.J. & CENDER D. (1998) A different look at corticosteroids. *Am Fam Physician.* 1998 Aug 1;58(2):443-450.

ZUCCHI, I., BINI, L., VALAPERTA, R., GINESTRA, A., ALBANI, D., SUSANI, L., SANCHEZ, J. C., LIBERATORI, S., MAGI, B., RAGGIASCHI, R., HOCHSTRASSER, D. F., PALLINI, V., VEZZONI, P. & DULBECCO, R. (2001). Proteomic dissection of dome formation in a mammary cell line: role of tropomyosin-5b and maspin. *Proc Natl Acad Sci U S A*, **98:** 5608-13.

ZUCCHI, I. & DULBECCO, R. (2002). Proteomic dissection of dome formation in a mammary cell line. *J Mammary Gland Biol Neoplasia*, **7:** 373-84.

ZUCCHI, I., MONTAGNA, C., SUSANI, L., MONTESANO, R., AFFER, M., ZANOTTI, S., REDOLFI, E., VEZZONI, P. & DULBECCO, R. (1999). Genetic dissection of dome formation in a mammary cell line: Identification of two genes with opposing action. *Proc Natl Acad Sci USA*, **96:** 13766-70.

Online References

Atlas of Human Embryology. <URL:http://www.embryo.chronolab.com/respiratory.htm> <accessed:5th June 2012>

Canadian Lung Association
<URL:https://www.lung.ca/children/grades7_12/respiratory/respiratory_system.html>
<accessed: 5th June 2012>